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THE SEPTUM AND THE AMYGDALA DIFFERENTIALLY MEDIATE THE ANXIOLYTIC EFFECTS OF BENZODIAZEPINES

BY

CHRISTINE PESOLD ©

A thesis submitted to the Faculty of
Graduate Studies and Research in partial
fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

DEPARTMENT OF PSYCHOLOGY

Edmonton, Alberta

FALL 1994

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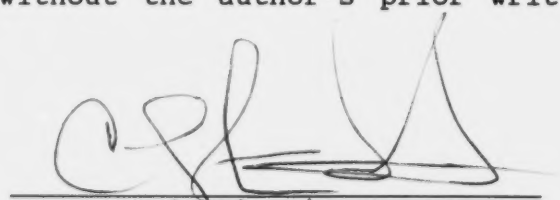

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled

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Dallas Treit
Dr. DALLAS TREIT

Dr. C. DONALD HETH

Dr. DOUGLAS WAHLSTEN

Dr. MATHEW T. MARTIN-IVERSON

Norm Stacey
Dr. NORMAN STACEY

Dr. JOHN P.J. PINEL

DATE: May 27, 1994

ABSTRACT

The goal of the present set of studies was to investigate the role of the septum and the amygdala in the mediation of the anxiolytic effects of benzodiazepines using two different animal models of anxiety: the elevated plus-maze and the shock-probe burying tests. The results of Experiment 1 showed that the septum and the amygdala differentially mediate the anxiolytic effects of midazolam i.e., intra-septal midazolam increased open-arm activity in the plus-maze, and decreased burying behavior in the shock-probe burying test, without affecting number of shocks received from the probe. Conversely, intra-amygdaloid infusions produced a dramatic increase in probe-contacts, without significantly affecting open-arm activity or burying behavior. Furthermore, each of these anxiolytic effects were antagonized by a pre-infusion of the benzodiazepine antagonist flumazenil. The results of Experiment 2 demonstrated that the anxiolytic effects of intra-septal infusions of midazolam are specific to the lateral septum since the effects of discrete infusions into this structure completely paralleled those of infusions into the entire septum (i.e., an increase in open-arm activity, and a decrease in shock-probe burying), while comparable infusions into the medial septum had no effect. Moreover, these midazolam-induced anxiolytic effects in the lateral septum were partially blocked by a pre-infusion of flumazenil. In Experiment 3, the anxiolytic effects of intra-amygdaloid

midazolam were further characterized i.e., infusions of midazolam into the basolateral nucleus produced a selective increase in open-arm activity in the plus-maze without affecting burying behavior or shock-probe avoidance in the shock-probe burying test, whereas infusions into the central nucleus produced a selective decrease in shock-probe avoidance, without significantly affecting burying behavior or open-arm activity. In addition, both of these distinct anxiolytic effects of midazolam in amygdala sub-nuclei were blocked by a pre-infusion of flumazenil. Taken together, these results suggest that the septum and the amygdala differentially mediate the anxiolytic effects of benzodiazepine anxiolytics. Furthermore, these results lend support to the concept that distinct types of fear responses to different types of fear stimuli may be differentially modulated by discrete brain regions.

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INTRODUCTION

Anxiety is a fundamental emotion that is characterized by an increase in autonomic activity, motor tension, vigilance, and apprehension in response to a present danger or impending threat (Wolkowitz & Paul, 1985). Anxiety can be considered pathological if it is inappropriate or exaggerated with respect to the anxiety-provoking stimulus (American Psychiatric Association, 1987; Lewis, 1967; Lader, 1983; Hommer, Skolnick, & Paul, 1987). These "anxiety disorders" have become increasingly prevalent in modern society, coinciding with an increased interest in understanding the biological basis of anxiety.

Many biological theories concerning the etiology of neuropsychiatric disorders have evolved from extrapolating neurochemical data on the mode of action of psychotropic agents. For instance, the "dopamine hypothesis of schizophrenia" and the "catecholamine theory of depression" have been developed from our understanding of the mechanism of action of drugs that either mimic or antagonize the symptoms of schizophrenia or depression, respectively (Bunney & Davis, 1965; Snyder & Yamamura, 1977). The benzodiazepine class of anti-anxiety drugs (i.e., anxiolytics), which are among the most widely prescribed drugs in the world, are not only effective in decreasing anxiety in the 'clinically' anxious, they also can decrease the characteristic symptoms of anxiety

in 'normal' volunteers [i.e., autonomic hyperactivity, motor tension, vigilance (Hommer et al., 1986)]. In this light, an elucidation of the precise neural mechanism of action of the benzodiazepine anxiolytics may enhance our knowledge of the neurochemical events underlying anxiety.

The Benzodiazepine Receptor and GABAergic Neurotransmission

In 1977, two independent research teams using radiolabelled [H^3]-diazepam, identified a stereospecific recognition site for benzodiazepines in the brain (Mohler & Okada, 1977; Braestrup & Squires, 1977). This benzodiazepine receptor site (Bzr) is now known to be closely associated with the naturally occurring neurotransmitter gamma-aminobutyric acid (GABA), which generally produces a post-synaptic inhibition of neural activity through a selective increase in chloride conductance (MacDonald & Barker, 1978). In addition, five types of benzodiazepine receptor ligands have been identified: agonists, inverse agonists, antagonists, as well as partial agonists and partial inverse agonists (Haefely, 1988). Benzodiazepine receptor agonists such as diazepam, midazolam, and chlordiazepoxide, bind to the benzodiazepine recognition site on the $GABA_A$ receptor subtype ($GABA_A$/Bzr), which then enhances the affinity of the $GABA_A$ receptor for its neurotransmitter GABA (Study & Barker, 1981). In other words, benzodiazepine receptor agonists exert their initial effects

by increasing the inhibitory actions of GABA. Substances such as the β-carbolines (e.g., β-CCE), in contrast, produce responses that are opposite to those of benzodiazepine receptor agonists and are hence termed inverse agonists (Braestrup, Schmieden, Nielsen, & Petersen, 1982). These substances attach to the same or overlapping site yet they decrease GABA-receptor affinity for its naturally occurring neurotransmitter. Benzodiazepine receptor antagonists, such as Ro 15-1788 (flumazenil) interact competitively with the benzodiazepine binding site to block the actions of both the receptor agonists and inverse agonists, but have no intrinsic effect on GABAergic function by themselves (Richards, Schoch, Mohler, & Haefely, 1986). The benzodiazepine receptor partial agonists and partial inverse agonists, as their name implies, only produce a partial enhancement or a partial inhibition of GABA-mediated chloride conductance, respectively (Haefely, Martin, & Schoch, 1990).

The GABAa/Benzodiazepine Receptor Complex and Anxiety

The different types of benzodiazepine receptor ligands exert different pharmacological effects in addition to anxiety modulation. Full benzodiazepine receptor agonists, such as diazepam, have anxiolytic, anti-convulsant, anti-aggressive, sedative, and muscle relaxant properties in both humans and other animals (Richards et al., 1986). Their potencies as

anxiolytics are correlated with their relative affinities for the benzodiazepine receptor binding site in both humans and other animals in vitro (Paul & Skolnick, 1981), as well as in vivo, using positron emission tomography and radiolabelled benzodiazepines (Comar, Maziere, Godot, Berger, & Soussaline, 1979; Maziere et al., 1981). The benzodiazepine receptor inverse agonists, such as FG 7142 and β-CCM, which produce effects opposite to those of agonists at the benzodiazepine receptor site, also possess opposite pharmacological properties. Full inverse agonists are anxiety-provoking (i.e., anxiogenic) in humans (Dorow, Horowsky, Paschelke, Amin, & Braestrup, 1983), as well as anxiogenic, convulsant, and pro-convulsant (i.e., lower convulsion threshold) in animals (Insel et al., 1984; Ninan et al., 1982). The effects that the different types of benzodiazepine receptor ligands have in modulating anxiety appear to be exerted through their actions at the benzodiazepine receptor site, since pure antagonists such as Ro 15-1788 (flumazenil) block both the anxiolytic effects of full agonists and the anxiogenic effects of inverse agonists (Haefely, 1983).

Functional Localization of the Benzodiazepine Receptor

There is growing evidence that benzodiazepine receptors exist in various forms, or functional subtypes, and are widely distributed in the central nervous system (CNS), with

particularly high concentrations being found in limbic system structures such as the amygdala, septum, and hippocampus (Mohler & Okada, 1978; McCabe & Wamsley, 1986; Speth et al., 1980; Young & Kuhar, 1980). Benzodiazepine full agonists such as diazepam, bind with high affinity to almost all of the benzodiazepine receptor subtypes, and produce a wide variety of pharmacological effects. Benzodiazepine receptor partial agonists such as zolpidem, which bind only to a sub-population of these receptors (Massotti et al., 1991), only produce part of the pharmacological profile of full agonists (Haefely et al., 1990). While the data are still somewhat limited, there is some evidence to suggest that the different pharmacological action of these $GABA_A$/Bzr partial agonists may be related to their location of action (Luddens & Wisden, 1991). For example, it may be the case that the sedative, ataxic, and muscle relaxant properties of benzodiazepines are a consequence of decreasing neuronal activity in some CNS regions [e.g., striatum, spinal cord (Guidotti et al., 1990)], and that their anxiolytic actions are a consequence of their ability to decrease neuronal activity in other brain regions (e.g., limbic system structures). If it can be assumed that the benzodiazepines' anti-anxiety effects are a consequence of their inhibitory action at particular brain structures, then it would follow that by identifying the structures at which these agents produce their **anxiolytic** effects, we may be identifying structures which are specifically involved in the

modulation of anxiety.

For a structure to mediate the anxiolytic effects of benzodiazepines, the following four criteria seem logical: 1) direct application of benzodiazepines into the structure should produce anxiolytic effects that are similar to those produced by peripheral administration, 2) the anxiolytic effects produced by direct application of a benzodiazepine into a structure should be antagonized by the co-administration of a benzodiazepine receptor antagonist, 3) lesions of the structure, or other physiological manipulations which would similarly decrease neuronal activity, should produce anxiolytic effects that are similar to those produced by the peripheral or central administration of benzodiazepines [this would follow from the presumed inhibitory role of benzodiazepine agonists on the neuronal activity of these structures], and 4) lesion of the structure should prevent, or at least diminish the anxiolytic effects of peripherally administered benzodiazepines, since the structure through which the drugs normally produce these effects would no longer be present. Since criterion 3 suggested that lesions should produce effects that are similar to those of anxiolytic drugs, criterion 4 may be difficult to satisfy for practical reasons (e.g., ceiling or floor effects associated with drug-lesion interactions). In any event, according to these criteria, there should be some parallels between the effects of

anxiolytic drugs and the effects of specific brain lesions, and therefore an examination of the lesion literature seems particularly important.

There is considerable research that has examined the role of brain structures in the modulation of experimental anxiety in animals. Two structures that have consistently been implicated in the modulation of anxiety are limbic system structures located in the forebrain of many animal species: the septum and the amygdala (for reviews, see Gray & McNaughton, 1983; and Davis, 1992a). In the following pages, this literature will be reviewed and the major problems with this research will be examined. It will be argued that these problems cast doubt on the putative roles of these structures in experimental anxiety. Finally, arguments in favour of an alternative approach to studying the involvement of the amygdala and the septum in 'anxiety' will provide the rationale for the conducted experiments.

Amygdala and Septal Lesions

Lesioning techniques include several means (e.g., electrical, chemical, aspiration, knife cut) of destroying particular brain structures. The assumptions underlying this approach is that the behavioral changes observed following lesions reflect the functions of the lesioned structures.

Therefore, for example, if a structure is involved in the expression of anxiety, then destruction of that structure should theoretically produce a disruption of normal anxiety reactions i.e., "anti-anxiety" effects. Anxiety itself typically has been inferred from the behavior of animals in a wide variety of experimental settings (e.g., conditioned avoidance; conditioned suppression), all of which involve an "aversive" or "fearful" stimulus and a "conditioned" or "unconditioned" fear response (Gray, 1982). Each of these experimental paradigms will be described in more detail in the following sections.

Amygdala. In conditioned avoidance paradigms, an animal is "conditioned" either to make a response (active avoidance), or to omit a response (passive avoidance) in order to avoid punishment (usually electric footshock). While lesions of the amygdala have been shown to have inconsistent effects on active avoidance, with reports of facilitation (e.g., Grossman, Grossman, & Walsh, 1975), no effect (e.g., King, 1958; Jellestad & Cabrera, 1986), or impairments (e.g., Campenot, 1969; Robinson, 1963), they have been shown to rather consistently attenuate response suppression in various passive avoidance tasks (e.g., Jellestad & Bakke, 1985; Lorenzini, Bucherelli, Giachetti, Mugnai, & Tassoni, 1991), including the conflict task [i.e., anti-conflict effect (e.g., Shibata, Kataoka, Yamashita, & Ueki, 1986; Shibata, Yamashita,

Yamamoto, Ozaki, & Showa, 1989)]. Amygdala lesions, however, were not able to block the anti-conflict effects of the benzodiazepine anxiolytic chlordiazepoxide (Yadin, Thomas, Strickland, & Grishkat, 1991).

Lesions of the amygdala have also been found to have profound effects on measures of conditioned fear. In these paradigms, the effects of presenting a signal that had been previously paired with punishment such as shock (i.e., "conditioned fear"), is measured on ongoing behavior. Similar to anxiolytic agents, lesions of the amygdala have been found to attenuate measures of conditioned fear including conditioned freezing (Jellestad & Bakke, 1985), conditioned cardiovascular responses (Gentile, Jarrell, Teich, McCabe, & Schneiderman, 1986), conditioned taste aversion (e.g., Kemble & Nagel, 1973; Nachman & Ache, 1974), conditioned emotional responding (Kellicut & Schwartzbaum, 1963; Spevak, Campbell, & Drake, 1975), and fear-potentiated startle (Hitchcock & Davis, 1986).

While the effects of amygdaloid lesions on passive avoidance learning and on "conditioned" fear are quite consistent, the effects of amygdaloid lesions on "unconditioned" fear reactions (i.e., fear reactions that do not involve prior training) have been a little more varied. For instance, while complete lesions of the amygdala did not appear to affect exploration of novel places (Becker, Walker, & Olton, 1980) or simple acoustic startle (Melia Sananes, &

Davis, 1991), amygdaloid-lesioned animals appeared to reduce anxiety in some situations i.e., tended to eat more readily in unfamiliar circumstances (Galef, 1970), and to increase anxiety in other situations i.e., decrease activity in the social interaction test (Jonason & Enloe, 1971).

Septum. The effects of septal lesions in conditioned active avoidance paradigms, like the effects of amygdaloid lesions, have also been quite varied. Septal lesions have been reported to impair (Thomas & Thomas, 1972), facilitate (e.g., Blatt, 1976; Carlson, 1970; Garber & Simmons, 1968; Poplawsky, 1978; Morgan & Mitchell, 1969; Sodetz, 1970, 1972; Zucher, 1965), or have no effect on active avoidance responding (Thomas & McCleary, 1974). Although septal lesions have generally been found to impair performance in simple (e.g., step-down) passive avoidance tasks (e.g., Beatty, Beatty, O'Brian, Gregoire, & Dahl, 1973; Bengelloun, Burright, & Donovick, 1977; McDaniel, Donovick, Burright, & Fanelli, 1980), they have inconsistent effects on response suppression measured in the "conflict task". Here lesions have been found to attenuate (e.g., Dickinson, 1975; Miczek, Kelsey, & Grossman, 1972), have no significant effect on (Yadin, Thomas, Grishkat, & Strickland, 1993; Yamashita et al., 1989), or enhance performance in conflict tasks (Yadin & Thomas, 1991). However, despite the equivocal effects of septal lesions on conflict behavior, these lesions have been shown to block the

anti-conflict effects of both benzodiazepine and non-benzodiazepine anxiolytics (Yadin, Thomas, & Strickland, 1987).

In contrast with amygdala lesions, the effects of septal lesions on conditioned fear have been rather equivocal. While septal lesions have been found to attenuate conditioned freezing (Duncan, 1971), they have been found both to attenuate (Harvey, Lints, Jacobson, & Hunt, 1965; Dickinson, 1975) or to have no effect (Brady & Nauta, 1955) on conditioned emotional responding. Also in contrast with the amygdaloid nucleus, the septal nucleus does not appear to be involved in the modulation of fear-potentiated startle, since septal lesions have been shown to neither affect fear-potentiated startle, nor to prevent the ability of benzodiazepine or non-benzodiazepine anxiolytics to decrease fear-potentiated startle (Melia & Davis, 1991).

Unlike the situation with amygdala lesions, however, there is reasonably consistent evidence that septal lesions may reduce unconditioned fear reactions. For instance, septal lesions, like anxiolytic agents, have been shown to increase levels of social interaction in rats (Clark & File, 1982). Septal lesions also reduce the latency to eat in a novel environment (Ross, Grossman, & Grossman, 1975), and reduce the emergence latency to go from a familiar to an unfamiliar place (Thomas, Moore, Harvey, & Hunt, 1959). The suppressive effects of novelty on eating, drinking, and emerging in novel

environments are also reduced following the administration of anti-anxiety agents (Soubrie, Kulkarmi, Simon, & Boissier, 1975; Soubrie, de Angelis, Simon, & Boissier, 1976; and Simon & Soubrie, 1979, respectively). However, septal lesions have been shown to increase simple acoustic startle, which can be considered a measure of unconditioned fear (Melia et al., 1991).

Summary. The effects of amygdaloid and septal lesions on conditioned avoidance are generally similar to those of anxiolytic agents i.e., they impair performance in passive avoidance tasks, but may facilitate, impair, or have no effect on active avoidance tasks. Amygdala and septal lesions appeared to have different effects on measures of conditioned and unconditioned fear, with amygdala lesions more consistently disrupting conditioned fear responses, while septal lesions more reliably reduce unconditioned fear responses. Therefore, while there is considerable evidence that both the amygdala and the septum are involved in the modulation of experimental anxiety in animals, the evidence suggests that these two structures may play very distinct roles in this modulation. Exactly what these roles are is unclear.

Amygdala and Septal Drug Infusions

There is a considerable amount of research that has examined the behavioral consequences of directly infusing anxiolytic drugs such as the benzodiazepines into specific brain structures. Generally, intracerebral infusions of anxiolytic drugs directly into the amygdala (and other limbic structures) produce effects that are similar to those produced by peripheral administration of these agents. Because most of the research that has examined the effects of benzodiazepine infusions into the amygdala has been investigated in the conflict paradigm (see Table 1 of Appendix for details), a more detailed description of the conflict paradigm will precede the discussion of this literature.

The several variants of the conflict paradigm all involve two components: the shock component and the non-shock component. In the non-shock component, a response (e.g., bar-pressing) is usually followed by a reward (e.g., food) on a variable interval schedule. In the shock component, the same behavior is both rewarded with food and punished by an aversive stimulus such as shock on a continuous reinforcement schedule (CRF), hence creating a 'conflict' between a richer reward schedule (CRF) and punishment. Normally, animals suppress responding in the punished or 'conflict' phase, restricting most of their responding to the non-punished or non-conflict phase. This shock-induced suppression of

responding is attenuated by the administration of anxiolytic agents such as benzodiazepine agonists (i.e., anti-conflict), and enhanced by the administration of anxiogenic agents such as benzodiazepine inverse agonists (i.e., pro-conflict). Furthermore, the benzodiazepine agonist and inverse agonist induced anti-conflict and pro-conflict effects can be blocked by the co-administration of benzodiazepine receptor antagonists (e.g., Geller & Seifter, 1960; Geller, 1962; Hunkeler et al., 1981; Bonetti et al., 1982).

Amygdala. Nagy and her colleagues (1979) were the first to report anti-conflict effects from relatively diffuse infusions of the benzodiazepine agonist, diazepam, into the whole amygdala. Since this initial report, Shibata and his colleagues observed anti-conflict effects following more discrete infusions of the benzodiazepines chlordiazepoxide, diazepam, and midazolam into the central nucleus, but not the medial or basolateral nuclei of the amygdala (Shibata, Kataoka, Gomita, & Ueki, 1982). These and other researchers later observed anti-conflict effects following central nuclei-infusions of GABA and the GABA mimetic muscimol (Kataoka, Shibata, Yomashita, & Ueki, 1987), the benzodiazepine anxiolytics flurazepam and lormetazepam (Higgins, Jones, Oakley, & Tyrers, 1991; Shibata et al., 1989), as well as non-benzodiazepine anxiolytics phenobarbital and zopiclone (Shibata et al., 1989). In this latter study, the anti-

conflict effects of diazepam and zopiclone were antagonized by the co-administration of the benzodiazepine antagonist Ro 15-1788 (flumazenil), as well as the benzodiazepine inverse agonist β-CCM, whereas the anti-conflict effects of phenobarbital were only antagonized by β-CCM (see Table 1 of Appendix).

In contrast to the results of Shibata and his colleagues, anti-conflict effects were observed following infusions of chlordiazepoxide (Thomas, Lewis, & Iversen, 1985), midazolam, diazepam, and muscimol (Scheel-Kruger & Petersen, 1982) into the lateral and basolateral nuclei, but not from infusions into the central nucleus. The anti-conflict action of midazolam, chlordiazepoxide, GABA, and muscimol appeared to be mediated via the GABA/benzodiazepine receptor: the effects of midazolam and muscimol were blocked from the pre-treatment with the GABA antagonist bicuculline (Scheel-Kruger & Petersen, 1982); the effects of midazolam were also blocked by the benzodiazepine antagonists Ro 15-1788 (flumazenil) and ZK 93426, as well as the benzodiazepine inverse agonists CGS 8216 and FG 7142 (Petersen, Braestrup, & Scheel-Kruger, 1985); the effects of chlordiazepoxide were also blocked by Ro 15-1788, CGS 8216, and FG 7142; and the effects of GABA were blocked by CGS 8216 and FG 7142, but not by Ro 15-1788 (Hodges, Green, & Glenn, 1987).

Anti-conflict effects were also observed following intra-amygdaloid infusions of anti-serotonergic agents such as

methysergide and cyproheptadine (Hodges et al., 1987; Kataoka et al., 1987), while pro-conflict effects were observed following intra-amygdala infusion of serotonin (Hodges et al., 1987). The mixed serotonin autoreceptor/benzodiazepine receptor agonist d-AP159, also produced an anti-conflict effect which was antagonized by systemic administration of Ro 15-1788 (Takao, Nagatani, Kasahara, & Hashimoto, 1992). The anti-conflict effect from drugs which decrease serotonin activity and the pro-conflict effect of serotonin itself are particularly interesting since drugs which interfere with serotonergic neurotransmission, either serotonin receptor antagonists such as mianserine, or serotonin autoreceptor agonists such as buspirone, have been shown to be clinically effective anxiolytic agents (Skolnick, Paul, & Weissman, 1984).

Intra-amygdaloid infusions of benzodiazepine agonists also appear to have anxiolytic effects on other behavioral indices of fear. For instance, intra-amygdaloid infusions of agonists such as diazepam have been shown to attenuate the fear-like sensitization process associated with the acoustic startle stimulus (Young, Helmstetter, Rabchenuk, & Leaton, 1991), to decrease defensive freezing (Helmstetter, 1993), and to decrease anxiety in the social interaction test when infused into the basolateral nucleus (Higgins, Jones, Oakley, & Tyers, 1991). Similarly, infusions of serotonin receptor antagonists into the basolateral amygdala have also been shown

to decrease anxiety in the social interaction test (Higgins et al., 1991).

Studies that have examined the effects of intra-amygdaloid infusions of benzodiazepines and other pharmacologically active agents suggest that the amygdala may also mediate some of the autonomic indices of fear. For instance, high doses of chlordiazepoxide and GABA infused directly into the central nucleus decreased stress-induced ulcerations, while benzodiazepine antagonist Ro 15-1788 potentiated stress-induced ulcerations (Sullivan, Henke, Ray, Hebert, & Trimpert, 1989).

Septum. While studies of the anxiolytic effects of intra-septal benzodiazepines are scarce, a few suggest that the septum may mediate anxiolytic drug effects. The septum may mediate some of the effects of serotonin anxiolytics, since decreasing serotonin activity in the septum appears to produce anxiolytic effects, while increasing serotonin activity may produce anxiogenic effects. For instance, intra-septal infusion of 8-OHDPAT (a 5-HT1 autoreceptor agonist) impaired passive avoidance responding, while intra-septal infusion of fluoxetine and zimelidine (5-HT re-uptake blockers) enhanced passive avoidance responding (Lee, Lin, Chen, Shiu, & Liang, 1992).

Summary. There is considerable evidence that the amygdala

may mediate some of the anxiolytic effects of benzodiazepines, as well as other putative anxiolytic agents. And while the intra-septal infusion research is scarce, it is not inconsistent with the view that the septum may also mediate some of the effects of anxiolytic agents.

Summary of Lesion and Drug-Infusion Research

Overall, the lesion research provides reasonable evidence that the amygdala and the septum are involved in the modulation of anxiety. The evidence even suggests that these two structures may be differentially involved in this control i.e., the amygdala appears to be involved in the modulation of "conditioned" fear responses, whereas the septum appears to be involved in the modulation of "unconditioned" fear reactions. However, these diverse behavioral tasks involve different types of fear responses to different types of fear stimuli, and it is unclear if the dissimilar effects of amygdala and septal lesions are a result of their distinctive roles in the modulation of anxiety, or rather an artifact of the behavioral requirements of particular test paradigms (e.g., measures of "conditioned" fear require learning and memory which may be more severely affected by lesions of the amygdala than by lesions of the septum).

The drug infusion research also suggests that the amygdala may be involved in the mediation of the anxiolytic

effects of benzodiazepines. However, these studies are rather limited to the effects of intra-amygdaloid infusions of benzodiazepines in the conflict paradigm, which only measures one type of fear reaction (i.e., response suppression) to one type of fear stimulus (i.e., electric shock). It is unclear, therefore, whether other fear responses to these or other fear stimuli would also be antagonized by intra-amygdaloid infusions of benzodiazepines, or whether other brain structures such as the septum would be similarly involved.

In addition to the scarcity of the critical research in this area, there are several, specific methodological problems that are associated with the lesion and drug-infusion techniques themselves. In the following sections these problems will be discussed, as well as problems associated with the measurement of 'anxiety' in non-human subjects.

Lesion and Drug-Infusion Techniques

Lesion. One problem in the lesion literature is the considerable amount of neuroanatomical variability between studies in the extent of damage to both the structure being examined, as well as damage extra to the intended target site. There is substantial evidence that differential damage could cause different behavioral results. For instance, lesions of the central nucleus of the amygdala appeared to attenuate

conflict-induced stomach ulcerations, while lesions of the ventromedial and anterolateral nuclei had no effect, and lesions of the posterolateral nucleus actually potentiated the gastric ulcers (Henke, 1980). Furthermore, electrolytic lesions (which form the bulk of the literature) not only destroy cell bodies in the target site, but they also destroy the fibers that pass through the area. It is therefore possible that the effects of the lesions are not specific or solely attributable to the destruction of the structure being examined. In some cases, electrolytic lesions produced effects that were different from those of neurotoxic lesions. For example, electrolytic or electrocoagulation lesions of the amygdala, which damage both cell bodies and fibers of passage, impaired passive avoidance, whereas these impairments were not reliably observed after neurotoxic lesions of the same structure (Jellestad, Markowska, Bakke, & Walther, 1986; Riobolos & Garcia, 1987, respectively).

Drug Infusion. While the amygdala drug-infusion research provides fairly consistent results, there are still some methodological questions that remain unanswered. For instance, while many studies showed that intra-amygdaloid infusion of benzodiazepine anxiolytics produced anti-conflict effects, few studies showed that these anti-conflict effects could be blocked by benzodiazepine receptor antagonists (e.g., Kataoka et al., 1987; Nagy et al., 1979; Shibata et al., 1982; Thomas

et al., 1985; Young et al., 1991). If the effects of the benzodiazepines are antagonized by the co-administration of a benzodiazepine antagonist, this would provide evidence that the behavioral effects of the benzodiazepines are actually mediated via the benzodiazepine receptor. It is therefore unclear from these studies whether the benzodiazepine effects actually resulted from a specific interaction of the benzodiazepine at its receptor site, as opposed to a non-specific effect on neural function. Furthermore, in studies that did use benzodiazepine antagonists, only one included a control group that received the antagonist alone, to determine if the antagonist was producing intrinsic effects of its own. The one study that did include such a control group in fact showed that the antagonist did produce intrinsic effects of its own (Hodges et al., 1987). Another problem with some of these studies stems from the use of water insoluble benzodiazepine agonists such as diazepam. In many studies, diazepam was dissolved in solvents such as polyethyleneglycol, ethanol (e.g., Nagy et al., 1979; Shibata et al., 1982; Shibata et al., 1989), or 100% DMSO (Young et al., 1991). The use of these solvents for central administration is problematic since they have been found to produce anti-conflict effects themselves (Petersen et al., 1985).

Behavioral Considerations

A different set of problems stems from the multiplicity of behavioral effects of amygdaloid and septal interventions. For example, there is evidence that amygdala and septal manipulations change general activity levels (e.g., Jellestad et al., 1986; Nielson, McIver, & Boswell, 1965; Corman, Meyer, & Meyer, 1967). Since changes in activity level may interact with the behavioral requirements in tasks which are supposed to reflect 'anxiety' (e.g., passive avoidance), such tasks may not be ideal for the study of these structures in anxiety. More importantly, many of these behavioral paradigms involve the use of food or fluid as appetitive reinforcers (e.g., conflict test). Since lesions of either the amygdala or the septum (e.g., Box & Mogenson, 1975; Donovick, Burright, & Gittelson, 1969), like benzodiazepine anxiolytics (Cooper, 1983) have profound effects on food and fluid consumption, behavioral paradigms that involve such reinforcers may not be appropriate for the study of the **anxiolytic** effects of amygdaloid and septal manipulations. Moreover, many of the tasks used to measure anxiety involve learning and remembering novel responses or associations (e.g., bar-pressing; shock signals). There is some evidence that amygdaloid and septal lesions may impair learning and memory (e.g., Cahill & McGaugh, 1990; Dunnett, 1985; Kelsey & Landry, 1988), and therefore tasks which strongly depend on the integrity of the

learning and memory systems may not be particularly suitable for the study of amygdaloid and septal manipulations on anxiety.

Another problem is that many of the putative indices of fear lack pharmacological corroboration i.e., clinically effective anxiolytic drugs should suppress the behavioral index of fear or anxiety in the test. With the exception of the 'conflict' test, many of the behavioral paradigms are not selectively sensitive to anxiolytic drugs. This lack of drug-class specificity in these paradigms raises the question of whether the measures of 'fear' used in these paradigms actually reflect fear or some other process. It should also be noted that most of these behavioral paradigms were not originally designed to study animal 'anxiety', but rather to study the "general laws of learning". Many of these tasks therefore require animals to make complex associations between arbitrary stimuli, or responses to situations that have little or no relevance to their natural repertoire of behavior. Animals appear to be 'phylogenetically prepared' to make associations between some stimuli, but not others. For instance, rats readily formed a "conditioned taste aversion" to a novel tasting solution when it was associated with sickness, but not when it was associated with shock (Garcia & Koelling, 1966). Since the neural substrates of 'phylogenetically prepared' fear reactions are more likely to reflect the neural substrates of anxiety, the use of animals'

untrained fear reactions to novel or painful stimuli may be more appropriate for the study of the mechanism of action of anti-anxiety agents.

An Alternative Approach

A more focused approach to studying the role of the septum and the amygdala in anxiety is to utilize behavioral paradigms that measure animals' untrained reactions to novel or painful stimuli, and that are selectively sensitive to anxiolytic agents. Two such tests are the elevated plus-maze test (Pellow, 1986), and the shock-probe burying test (Treit, Pinel, & Fibiger, 1981).

In the elevated plus-maze, two arms are open, and two arms are enclosed with walls. On first exposure, rats normally avoid the open arms of the maze, restricting most of their activity to the closed arms. This avoidance of the open arms is likely to reflect 'anxiety' since rats display behavioral and physiological signs of 'fear' when confined to the open arms [e.g., increased freezing, immobility, defecation, and plasma corticosterone levels (Pellow, Chopin, File, & Briley, 1985)]. Open-arm activity in the plus-maze is defined as the amount of time that the rat spends in the open arms relative to the total time in any arm (i.e., % open/total), as well as the number of entries into the open arms relative to the total number of entries into any arm (i.e., % open/total). General

activity is measured as the total number of entries into all arms. The relatively low level of open-arm activity (10-20%), is the primary measure of 'anxiety' in this paradigm. Anxiolytic agents such as diazepam, chlordiazepoxide, phenobarbitone, and tracazolate selectively increase open-arm exploration (Pellow et al., 1985), while putative anxiogenic agents such as yohimbine, amphetamine, caffeine, and pentylenetetrazol (PTZ) suppress rats' open-arm activity below baseline control levels (Pellow & File, 1986). Furthermore, non-anxiolytic agents including antidepressants (e.g., imipramine and mianserine) and major tranquilizers (e.g., haloperidol) have no specific effects on open-arm activity (Pellow et al., 1985).

In the shock-probe burying paradigm, rats shocked once from an electrified probe mounted on the wall of a test chamber will characteristically spray bedding material from the floor of the chamber toward the probe with rapid alternating movements of the forepaws. This "burying" behavior has been observed toward a variety of aversive or 'threatening' stimuli such as airblasts, physical blows, light flashes (Terlecki, Pinel, & Treit, 1979), noxious smells (Pinel, Gorzalka, & Ladak, 1981), and fluids previously paired with toxicosis (Wilkie, MacLennan, & Pinel, 1979). Rats appear to rapidly associate the shock or other aversive stimulus with a spatially contiguous cue (e.g., the probe), and following first exposure to shock, proceed to bury the source of the

aversive stimulus with whatever material is present, e.g., bedding, sand, or wooden blocks (Pinel & Treit, 1979). At the same time, rats show increased plasma corticosterone and adrenaline, and will avoid further contact with the probe (De Boer, Slangen, & Van der Gugten, 1990; Treit & Fundytus, 1988). Burying behavior is the major index of 'anxiety' in this test (Treit et al., 1981), and is dose-dependently suppressed by standard anxiolytic agents such as diazepam, chlordiazepoxide, and pentobarbital (Treit et al., 1981), and enhanced by anxiogenic agents such as yohimbine and beta-CCE (Tsuda, Yoshishige, & Tanaka, 1988; Tsuda, Ida, Nishimura, & Tanaka, 1989). Other non-anxiolytic agents such as imipramine, morphine, d-amphetamine, pentylenetetrazol, and picrotoxin do not produce specific effects on burying behavior. In addition to suppressing burying behavior, anxiolytic drugs antagonize shock-probe avoidance and block shock-induced increases in corticosterone and adrenaline (e.g., De Boer et al., 1990; Treit, 1990; Treit & Fundytus, 1988).

These behavioral and pharmacological characteristics suggest that the elevated plus-maze and the shock-probe burying paradigm may be useful for studying the role of the septum and the amygdala in the mediation of the anxiolytic effects of benzodiazepines. Both major indices of 'anxiety' in these tests (i.e., open-arm avoidance and burying behavior) appear to make minimal demands on learning and memory processes i.e., both fear responses are expressed, without

prior training, in the presence of the 'fearful' stimulus. Furthermore, neither of these tests are complicated by changes in appetitive motivation since neither involve food or fluid reward. The combined use of these two tests may be particularly advantageous since they measure different types of fear reactions to different types of fear stimuli i.e., in the plus-maze test, fear is primarily indicated by rats' passive avoidance of novel, elevated, open platforms (open-arm avoidance), while in the shock-probe burying test, fear is primarily indicated by active "burying" of a spatially discrete painful stimulus (shock-probe), and secondly by passive avoidance of contact-induced probe-shocks. Moreover, an anxiolytic effect in the elevated plus-maze is primarily indicated by an <u>increase</u> in a specific behavior (open-arm activity), whereas an anxiolytic effect in the shock-probe burying test is indicated by both a <u>decrease</u> in one specific behavior (probe-burying), and an <u>increase</u> in another (shock-probe contacts). For these reasons, anxiolytic profiles observed in both these tests following pharmacological manipulation of the septum or the amygdala would be difficult to explain in terms of non-specific effects on general activity or arousal.

Recent studies in our laboratory have found that electrolytic lesions of the septum and the amygdala produce distinct anxiolytic profiles in these tests. Septal lesions

increased open-arm activity in the elevated plus-maze and decreased burying behavior in the shock-probe burying test, without producing any systematic effects on general activity, handling reactivity, or shock reactivity (Treit & Pesold, 1990). Subsequent studies revealed that these effects were anatomically specific since they occurred following lesions to the posterior septum, but not the anterior septum (Treit & Pesold, 1990). Excitotoxic lesions of the septum produced a pattern of anxiolytic effects that were similar to those produced by electrolytic lesions of the same area with one exception: kainic acid and quisqualic acid lesions, like anxiolytic drugs, also antagonized rats' shock-probe avoidance (Pesold & Treit, 1992).

Lesions of the amygdala, in contrast, did not significantly affect open-arm activity on the elevated plus-maze (Treit, Pesold, & Rotzinger, 1993a, 1993b) nor did they suppress burying behavior in the shock-probe burying test (Treit et al., 1993a, 1993b; Kopchia, Altman, & Commissaris, 1992; Roozendaal, Koolhaas, & Bohus, 1991). Electrolytic lesions of the amygdala, like anxiolytic drugs, did however increase rats' contacts with the electrified probe (Treit et al., 1993a, 1993b). Further experiments showed that each of the distinct anxiolytic effects of septal and amygdaloid lesions occurred simultaneously in animals with combined lesions to both these structures (Treit et al., 1993a) suggesting that these two structures independently control the

expression of these different fear-related behaviors. Finally, there are some preliminary data indicating that the septum may be involved in the mediation of some of the anxiolytic effects of benzodiazepines in these tests, since infusions of diazepam into the lateral septum decreased burying behavior, and central infusions of a benzodiazepine receptor antagonist (Ro 15-1788) were able to block the effects of peripherally administered diazepam (Treit, 1991). It is also noteworthy that intra-amygdaloid infusions of serotonin receptor antagonists have been reported to decrease anxiety in the elevated plus-maze test (Tomkins, Costall, & Kelly, 1990).

In view of these results, the following set of experiments was designed to examine further the roles of the septum and the amygdala in mediating the anxiolytic effects of benzodiazepines, using the elevated plus-maze and the shock-probe burying tests as animal models of 'anxiety'.

GENERAL METHODS

Subjects

The subjects were naive, male albino Sprague-Dawley rats purchased from Charles River, Canada. Following surgery, rats were individually housed in polycarbonate cages and maintained on a 12-hour light/dark cycle (lights on at 0700), with food and water available ad lib. Behavioral testing occurred between 0900 and 1800 hr.

Apparatus

Plus-Maze. This apparatus was a wooden plus-shaped maze, elevated to a height of 50 cm, consisting of two 50 X 10 cm open arms, and two 50 X 10 X 50 cm enclosed arms, each with an open roof. The testing room was quiet and dimly lit at the time of testing. Behavior was observed via a mirror suspended at an angle above the maze. Behavioral data were collected by an observer who sat quietly one meter behind one of the closed arms of the maze.

Shock-Probe Burying. The shock-probe burying apparatus was in a separate, quiet testing room. The floor of this 40 X 30 X 40 cm plexiglass chamber was evenly covered with 5 cm of bedding material (odour-adsorbent kitty litter). On one of the

walls of the plexiglass chamber, 2 cm above the bedding material, was a small hole through which the probe could be inserted. The 6.5 cm X 0.5 cm diameter probe was helically wrapped with two copper wires through which electric current was administered. Shock intensity was adjusted with a variable resistor in series with a 2000 V shock source, and set at 2 Ma. The behavior of each rat was recorded on video tape via closed circuit television, and was later measured by an observer who was unaware of the surgical history and pharmacological treatment of the rats.

Surgery

The animals were anaesthetized [20 mg/kg pentobarbital (i.p.) and 60 mg/kg ketamine (i.m.)] and placed individually in a Kopf stereotaxic instrument. In each experiment, half of the rats were implanted with guide cannulae (26 gauge stainless steel, from Plastics One, USA) in one of two structures, while the other half of the rats were implanted with guide cannulae in the other one of two structures (Experiment 1: septum or amygdala; Experiment 2: medial or lateral septal nuclei; and Experiment 3: central or basolateral amygdaloid nuclei). Each cannula was surrounded by three skull screws and secured in place using dental cement.

Histology

At the conclusion of behavioral testing, rats were given an overdose of chloral hydrate, infused with 0.01 μl of a concentrated thionine solution to mark the location of the cannula tip, and perfused intracardially with physiological saline followed by 10% formalin. Brains were extracted and placed in 10% formalin for a minimum of three days, after which they were frozen and sectioned using a cryostat. To determine the exact location of the cannula tip, every fourth 32 μm section in the area of the intended target structure was taken, mounted on a slide, stained with neutral red and counter-stained with Luxol fast blue, and examined microscopically. An observer blind to the behavioral results, determined the locations of the cannulae tips microscopically by examining the pattern of gliotic cells and thionine stain surrounding the end of the cannulae tracts. Data from animals in which cannulae were not located in the intended target site were discarded. The location of the cannulae tips were transcribed onto the appropriate Paxinos and Watson (1986) atlas plates.

Drugs

Flumazenil (Ro 15-1788 donated by Hoffman LaRoche, Switzerland) was suspended in vehicle (0.9% saline plus 1%

Tween 80), using a vortex Gemini 2 mixer (Fisher Scientific), at a concentration of 15.0 μg/μl. Midazolam maleate (donated by Hoffman LaRoche, Switzerland) was dissolved in 0.9% saline at a concentration of 10.0 μg/μl. Optimal drug concentrations and volumes were determined on the basis of a series of pilot studies. All animals were given a drug pre-treatment of either flumazenil or vehicle, followed ten minutes later by a drug treatment of either midazolam or 0.9% saline.

Procedures

Handling. Handling consisted of a mild, hand-restraint for a minimum of three minutes during which time cannulae obturators were removed and cleaned to habituate the animals to the type of mild restraint and cannula manipulation that followed on test days.

Drug Pre-Treatment. Half of the hand-held awake animals were given an infusion of flumazenil, and half were given an infusion of vehicle. Flumazenil or vehicle were infused at a rate of 1.0 μl/min through a 33 gauge stainless steel internal cannula, lowered to 1.0 mm below the tip of the guide cannula. The cannula was connected via polyethylene tubing to a 50 μl Hamilton microsyringe, and was left in place an additional minute to allow the drug to diffuse away from the cannula tip.

Drug Treatment. Ten minutes after drug pre-treatment, half of the flumazenil pre-treated animals received an infusion of midazolam, and the other half received an infusion of saline. Similarly, half of the vehicle pre-treated animals were infused with midazolam, while the other half were infused with saline. Infusion and diffusion procedures were the same as those for drug pre-treatment. Animals were tested three minutes following drug treatment.

Plus-Maze. Following four consecutive days of handling, each rat was given its respective drug pre-treatment and treatment regimen, and individually placed in the center of the plus-maze (12 days post-surgery). An observer measured time spent in the open arms, time spent in the closed arms, number of entries into the open arms, and number of entries into the closed arms, for a five minute test period. An entry was defined as all four paws in the arm. For the purpose of analysis, open-arm activity was quantified as the amount of time that the rat spent in the open arms relative to the total amount of time spent in any arm (open/total X 100), as well as the number of entries into the open arms relative to the total number of entries into any arm (open/total X 100). The maze was cleaned after each rat was tested.

Shock-Probe Burying. On the eighteenth day post-surgery, rats were handled and habituated to the plexiglass test

chamber for 20 minutes, for the first of four consecutive days. On the fifth day (22 days post-surgery), rats were placed in the chamber containing the constantly electrified probe, which was inserted 6 cm into the chamber just prior to the test. When the rat touched the constantly electrified probe with its snout or forepaws, it received a brief, electric shock. Following the first shock, the duration of time each rat spent spraying bedding material toward the probe (i.e., burying behavior) was measured for fifteen minutes, as well as the total number of shocks each rat received from the probe. The rat's behavioral reaction to each shock was measured on the following four point scale: 1) flinch involving only head or forepaws, 2) whole body flinch and ambulation to far end of chamber, 3) hopping away and running, 4) jumping (all feet in the air) and running. A mean shock reactivity score was derived for each rat by summing their reactivity scores to each shock, and dividing this by the number of shocks obtained. In order to assess drug effect on general activity, the total time that the rat spent immobile (i.e., resting or freezing) during the 15 minute test was measured. To minimize variation in the infusion-test interval, any rat that could not be shocked within five minutes was removed from the chamber and excluded from shock-probe testing.

Ataxia. Immediately following both the plus-maze and the

shock-probe tests, possible drug effects on ataxia were assessed by measuring each rat's ability to remain for 30 seconds on a wire-mesh screen, inclined 70 degree.

Statistical Analysis

All three experiments consisted of eight groups: the two structures being compared, each with four different drug treatments: vehicle/saline, vehicle/midazolam, Ro 15-1788/saline, Ro 15-1788/midazolam. In the first experiment, planned contrasts (alpha=0.05), which compared the septal vehicle/midazolam group against the other seven groups, were performed to determine if this group differed from all other groups on the following two measures: percentage of time spent and percentage of entries into the open arms. Similarly, a planned contrast (alpha=0.05) which compared the amygdaloid vehicle/midazolam group against the other seven groups was performed to determine if this group differed from all others on the number of shocks received from the probe. An analysis of the variance between the other seven groups was performed to determine if these latter groups differed significantly from each other. A between groups one-way analysis of variance was performed on the eight groups for all other measures except duration of burying (alpha=0.05).

In Experiments 2 and 3, a between groups one-way analysis of variance (alpha=0.05) was performed on the eight groups,

for all measures (except burying behavior). These analyses were followed by pair-wise comparisons, where appropriate (Newman-Keuls, alpha=0.05).

The burying data in the three experiments, which were not normally distributed, were analyzed with Kruskal-Wallis one-way analyses of variance with correction for tied ranks (alpha=0.05), followed by Mann-Whitney U pair-wise comparisons (alpha=0.05).

Experiment 1

The first purpose of the present experiment was to determine if the septum or the amygdala are involved in the mediation of the **anxiolytic** effects of benzodiazepines. If the septum and the amygdala mediate at least some of the anxiolytic effects of benzodiazepines, then direct intracerebral infusion of MDZ into these structures should produce anti-anxiety effects in the plus-maze or shock-probe tests. Furthermore, if the behavioral effects of intracerebral infusions of midazolam into these structures are mediated via the $GABA_A$/benzodiazepine receptor, then these effects should be antagonized by the pre-administration of a benzodiazepine receptor antagonist (Ro 15-1788). The second purpose of this experiment was to determine if these two structures play similar or different roles in the mediation of anxiolytic drug effects. If the septum and the amygdala are differentially involved in the mediation of the anti-anxiety effects of these agents, then different anxiolytic profiles would be expected to emerge following the direct infusion of a benzodiazepine agonist into these two structures.

METHOD

The methods in Experiment 1 were essentially the same as those described in the General Methods with the exception of the surgical coordinates employed, and the volume of drug infused.

Subjects

The subjects were 124 male, albino Sprague-Dawley rats, weighing 275-345 g at the time of surgery. Housing and feeding conditions were the same as those described in the General Methods section.

Surgery

Using flat skull coordinates, 62 rats were implanted with a guide cannula position 1.0 mm above the middle of the septal area (0.7 mm anterior and 0.4 mm lateral to bregma, 3.0 mm ventral to dura, with the cannula angled 4 degrees medially to avoid the sagittal sinus), and 62 rats were bilaterally implanted with guide cannulae positioned 1.0 mm above the middle of the amygdaloid complex (2.4 mm posterior and 4.4 mm lateral to bregma, 6.0 mm ventral to dura).

Procedures

Drug Pre-Treatment. Half of the septal-implanted animals were given a 1.0 μl infusion of flumazenil, and half were given a 1.0 μl infusion of vehicle. Half of the amygdala-implanted animals were given 1.0 μl/side bilateral infusions of flumazenil, and the other half were given 1.0 μl/side bilateral infusions of vehicle. Infusion and diffusion rates were the same as those described in the General Method section.

Drug Treatment. Ten minutes after drug pre-treatment, half of the flumazenil pre-treated animals received a 1.0 μl infusion of midazolam, and the other half received a 1.0 μl infusion of saline. Similarly, half of the vehicle pre-treated animals were bilaterally infused with 1.0 μl of midazolam, while the other half were bilaterally infused with 1.0 μl of saline.

RESULTS AND DISCUSSION

Histologies

The location of the cannulae tips for the four groups of septally-implanted rats are illustrated by black dots in Figure 1. The behavioral data from animals in which cannulae

were located outside of the septal region (n=12) were discarded leaving 12 rats in the vehicle/saline group (VEH-SAL), 15 rats (14 in plus-maze) in the vehicle/midazolam group (VEH-MDZ), 11 rats in the Ro 15-1788/saline group (Ro-SAL), and 12 rats in the Ro 15-1788/midazolam group (Ro-MDZ).

Black dots represent the location of the cannulae tips for the four groups of amygdala-implanted rats in Figure 2. The behavioral data from an animal with a cannula located outside of the amygdaloid complex were discarded leaving 15 rats in the VEH-SAL group, 17 rats in the VEH-MDZ group, 14 rats in the Ro-SAL group, and 15 rats (14 in shock-probe) in the Ro-MDZ group.

Plus-Maze

The mean percentage of time spent in the open arms by the septal- and the amygdala-infused rats are shown in Figure 3, and their mean percentage of entries into the open arms are shown in Figure 4. It is apparent that infusions of midazolam into the septum produced an increase in rats' open-arm activity compared to controls, which was blocked by the pre-infusion of Ro 15-1788. It is also apparent that open-arm activity was not affected by midazolam, Ro 15-1788, or their combination when infused into the amygdala.

Planned comparisons confirmed that the animals that had received midazolam in the septum (VEH-MDZ) spent significantly more time in the open arms than the other seven groups

($t(102)=6.584$; $p<0.001$), while these latter seven groups did not differ significantly from each other [$F(6,89)=0.883$; $p>0.50$). Similarly, animals that received midazolam (VEH-MDZ) in the septum showed a significant increase in the percentage of open-arm entries compared to the other seven groups ($t(102)=5.616$; $p<0.001$), these latter seven groups not differing significantly from each other [$F(6,89)=1.568$; $p>0.10$]. The anxiolytic effects of midazolam in the septum could not have been attributable to any non-specific changes in general activity, since there were no significant differences among any of the groups in terms of total number of arm entries [$F(7,102)=1.78$, $p>0.1$; see Table 2].

These results suggest that the septum, but not the amygdala, mediate the anxiolytic effects of the benzodiazepine agonist midazolam in the elevated plus-maze. Furthermore, the blockade of these effects by a pre-infusion of the benzodiazepine receptor antagonist Ro 15-1788, suggests that these anxiolytic effects are being mediated via the benzodiazepine receptor.

Shock-Probe Burying

The mean duration of time that the septal- and the amygdala-infused animals spent burying the probe is shown in Figure 5. Significant Kruskal-Wallis analysis of variance ($H_{corr}=15.70$; $p<0.05$) followed by Mann-Whitney U pair-wise comparisons (alpha=0.05) confirmed that midazolam infused into

the septum (VEH-SAL) significantly decreased rats' probe-burying behavior, whereas midazolam infused into the amygdala did not. In addition, this anti-fear effect of intra-septal infusions of midazolam was antagonized by the pre-infusion of Ro 15-1788 (Mann-Whitney U=55, p=0.05). Pair-wise comparisons of the duration of time rats in each group spent burying the shock-probe showed that only animals that received midazolam into the septum (VEH-MDZ) buried the probe significantly less than their respective controls (VEH-SAL). Furthermore, the amount of burying in the septal midazolam group was significantly lower than all other groups, while these did not differ significantly from each other (Mann-Whitney U tests, alpha=0.05). These results are consistent with those in the plus-maze suggesting that the septum, but not the amygdala, mediates the anti-fear effects of midazolam.

However, an examination of the shock-probe contact results revealed that this generalization is not entirely correct. As shown in Figure 6, infusions of midazolam into the amygdala, but **not** the septum, dramatically increased the number of contact-induced shocks received from the probe, which was antagonized by the pre-infusion of Ro 15-1788. Planned contrasts confirmed that rats that had received midazolam in the amygdala (VEH-MDZ) received significantly more shocks than the other seven groups ($t(102)=9.858$, $p<0.001$), while these latter seven groups did not differ significantly from each other [$F(6,88)=0.623$; $p>0.50$]. This

selective increase in probe-shocks did not appear to be due to a decrease in pain sensitivity because there were no significant differences among the groups in their reactivity to shock [$F(7,102)=0.93$, $p>0.5$; see Table 3]. These results suggest that the amygdala, but not the septum, specifically mediates the anxiolytic effects of midazolam on shock-probe avoidance.

Analysis of variance revealed that there were significant differences in general activity levels in these groups of rats [$F(7,102)=4.97$, $p<0.0002$; see Table 3]. Pair-wise comparisons confirmed that animals that had received midazolam in the septum (VEH-MDZ), as well as animals that had received a combination of the two drugs in the amygdala (Ro-MDZ), had spent significantly more time immobile than their respective controls. However, since the VEH-MDZ septal-infused rats showed a decrease in burying and an increase in open-arm exploration, while the VEH-MDZ amygdala-infused rats showed no change in open-arm exploration or burying but an increase in shock-probe contacts, it seems unlikely that drug-induced changes in general activity levels can account for the overall pattern of behavioral results.

Taken together, these results suggest that the septum and the amygdala play separate roles in the mediation of the anxiolytic effects of benzodiazepines in the shock-probe burying test.

Ataxia

In contrast to peripheral injections of moderate to high doses of benzodiazepines (e.g., 5 mg/kg diazepam), central infusions of midazolam, Ro 15-1788, or their combination in this experiment failed to produce measurable ataxia in any of the animals i.e., none fell off the inclined screen during the 30 second test.

Figure 1

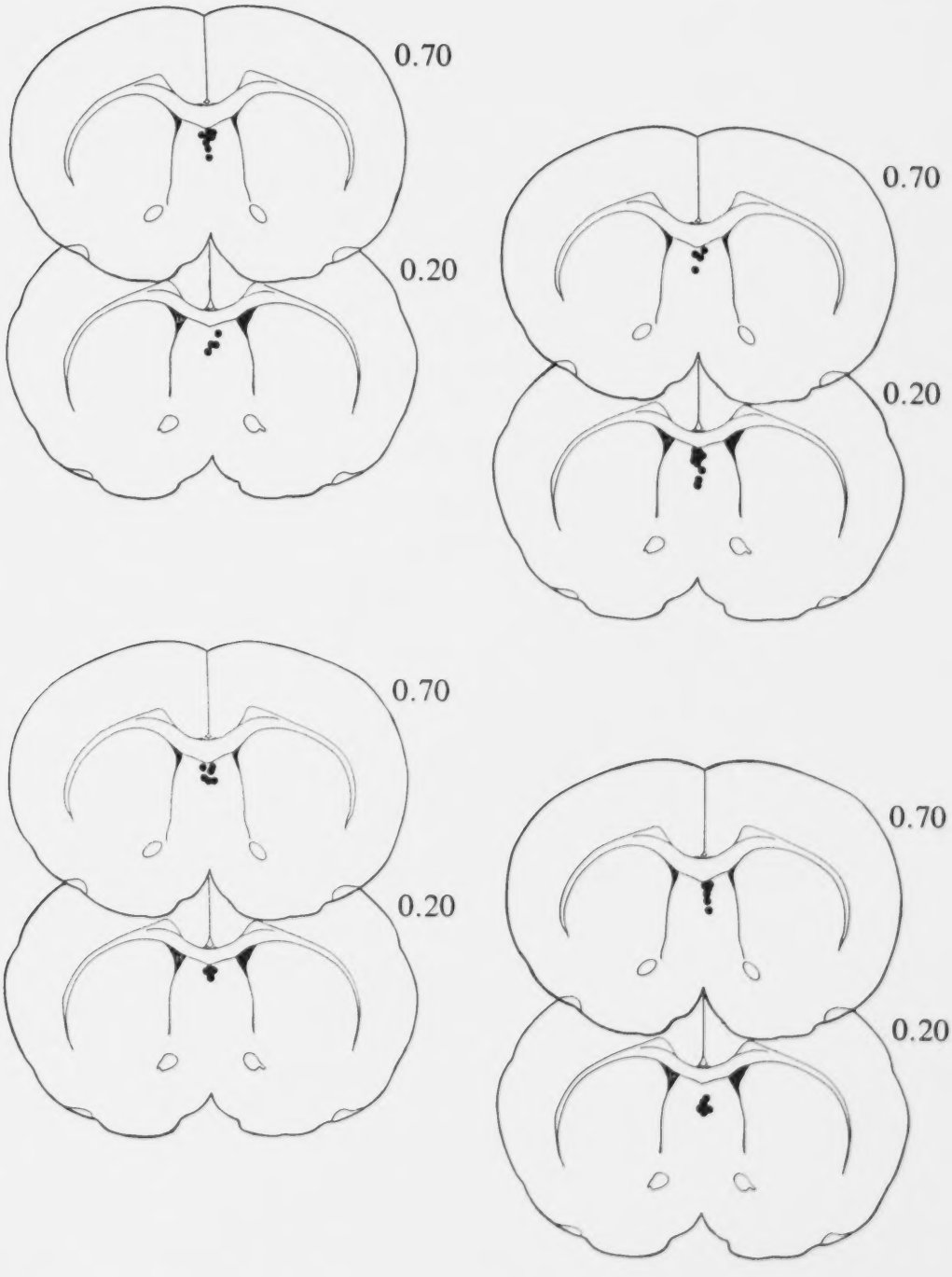
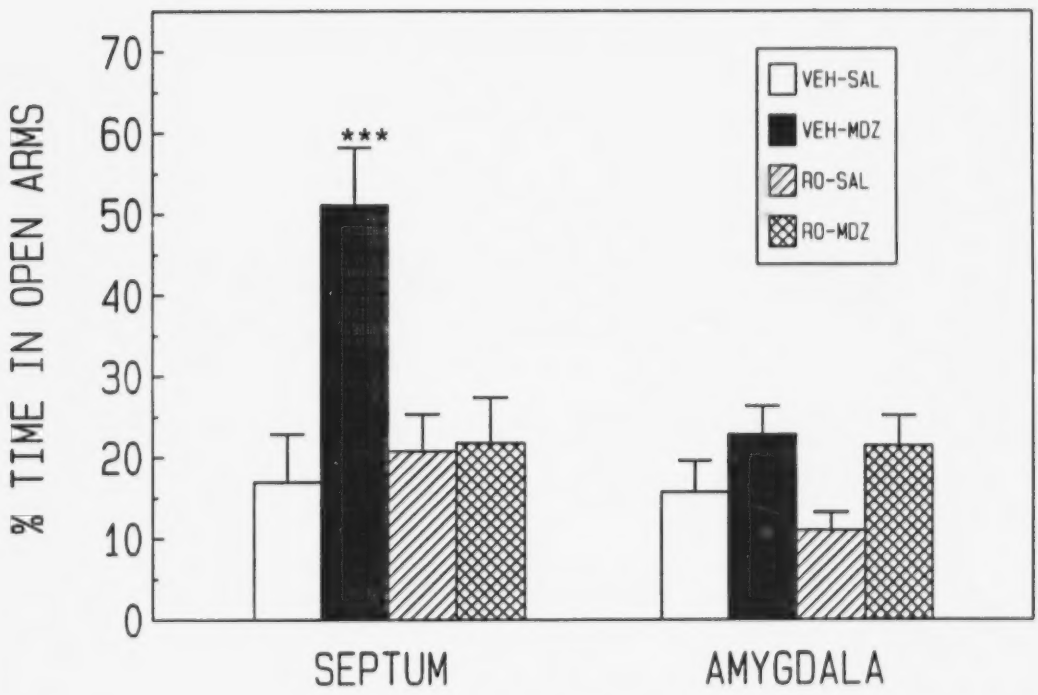


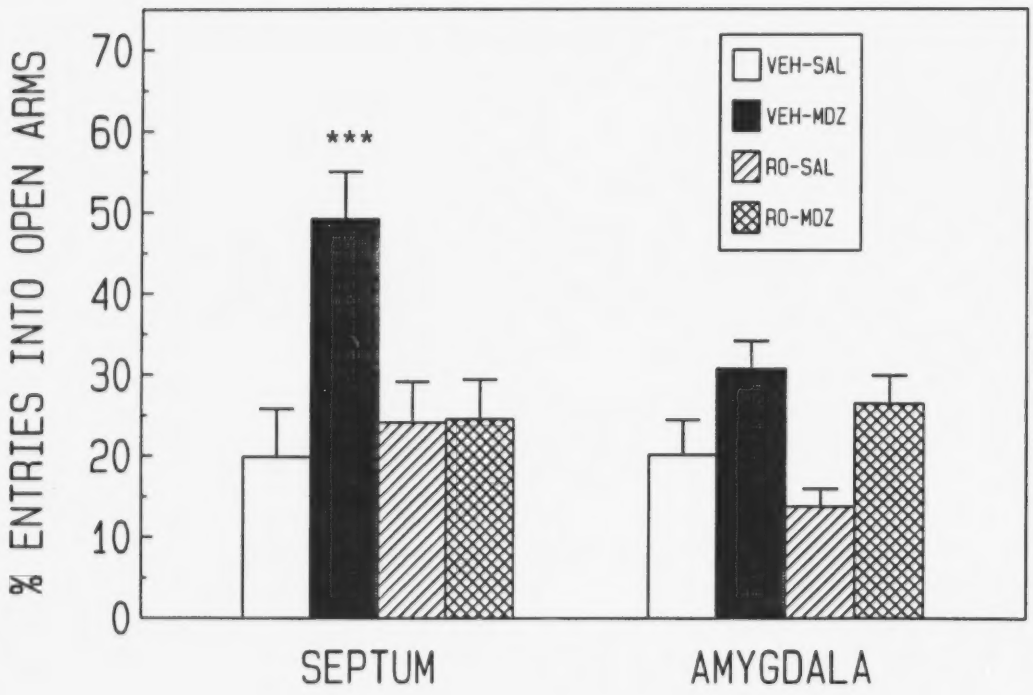
Figure 2

Figure 3



* p<0.05 compared to controls
** p<0.01 compared to controls
*** p<0.001 compared to controls

Figure 4



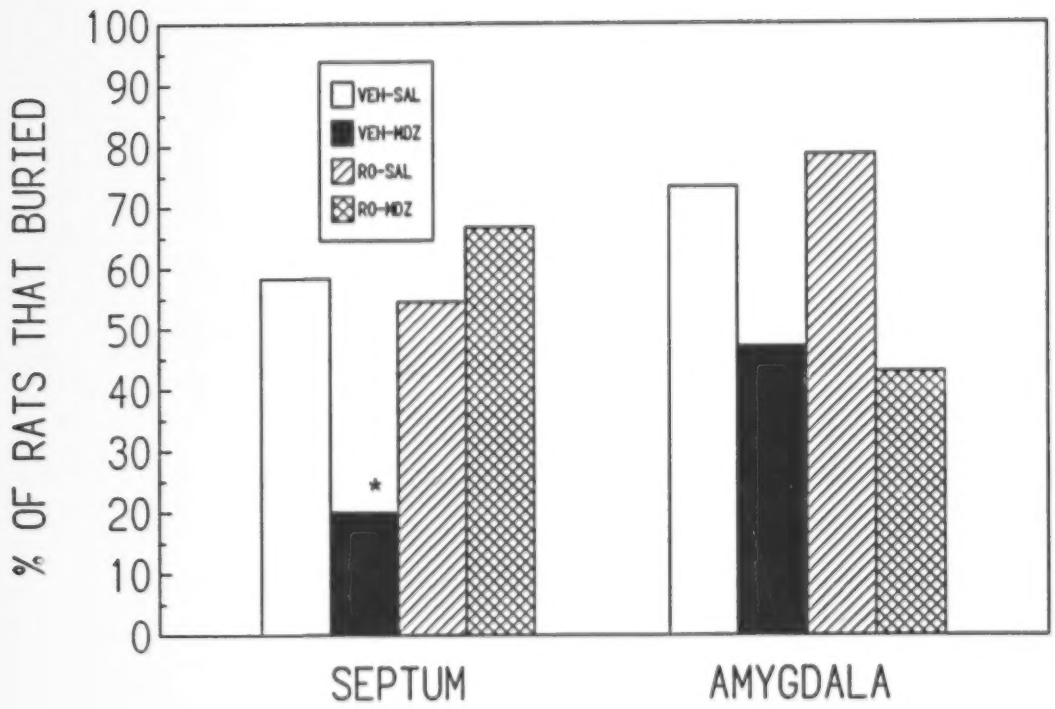
* p<0.05 compared to controls
** p<0.01 conpared to controls
*** p<0.001 compared to controls

Table 2

		Total Number of Arm Entries
SEPTUM	VEH-SAL (n=12)	12.17 (0.83)
	VEH-MDZ (n=14)	15.07 (1.23)
	Ro-SAL (n=11)	12.91 (0.67)
	Ro-MDZ (n=12)	15.58 (1.29)
AMYGDALA	VEH-SAL (n=15)	13.07 (0.80)
	VEH-MDZ (n=17)	14.65 (0.79)
	Ro-SAL (n=14)	13.21 (0.92)
	Ro-MDZ (n=15)	15.73 (1.29)

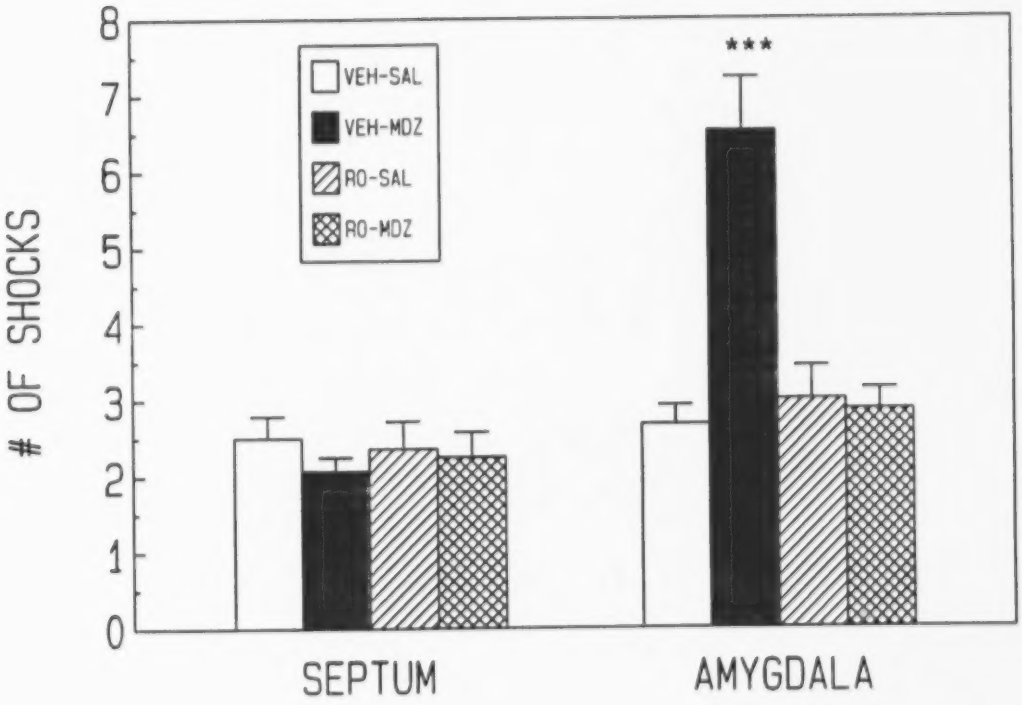
* $p<0.05$ compared to controls
** $p<0.01$ compared to controls

Figure 5



* p<0.05 compared to controls
** p<0.01 conpared to controls

Figure 6



* p<0.05 compared to controls
** p<0.01 compared to controls
*** p<0.001 compared to controls

Table 3

		Shock Reactivity	Immobility (sec)
SEPTUM	VEH-SAL (n=12)	1.83 (0.10)	95.17 (35.41)
	VEH-MDZ (n=15)	1.93 (0.11)	286.2 * (63.98)
	Ro-SAL (n=11)	2.06 (0.16)	125.82 (30.21)
	Ro-MDZ (n=12)	1.94 (0.06)	190.83 (45.85)
AMYGDALA	VEH-SAL (n=15)	1.91 (0.06)	40.27 (7.97)
	VEH-MDZ (n=17)	1.75 (0.09)	167.59 (36.58)
	Ro-SAL (n=14)	1.82 (0.12)	91.14 (16.31)
	Ro-MDZ (n=14)	1.97 (0.11)	278.5 ** (52.92)

* $p<0.05$ compared to controls
** $p<0.01$ compared to controls

Experiment 2

The results from the first experiment revealed that the septum mediates some of the anxiolytic effects of the benzodiazepine agonist midazolam. Intra-septal midazolam increased open-arm activity in the elevated plus-maze and decreased burying behavior in the shock-probe burying test, without affecting the number of shocks received from the probe. Furthermore, these anxiolytic effects likely occurred at the benzodiazepine receptor since they were found to be blocked by a pre-infusion of the benzodiazepine receptor antagonist Ro 15-1788.

However, the septum has two major subdivisions rostral to the fornix, the lateral and the medial septal nuclei, each with distinct efferent and afferent projections. Previous studies have suggested that different septal nuclei may play different roles in the modulation of 'anxiety'-related behaviors. For instance, studies that have compared the effects of medial and lateral septal lesions have found that lesions of these two subnuclei produce very different effects on "anxiety"-related behaviors including startle (Lee, Lin, & Yin, 1988), social behavior (Poplawsky & Johnson, 1973), reactions to novelty (Myhrer, 1989), and avoidance learning (Hamilton, Kelsey, & Grossman, 1970). Previous experiments in our laboratory have suggested that the anti-fear effects of septal lesions were restricted to the posterior septum since

lesions of the posterior, but not the anterior septum, produce anti-fear effects in both the elevated plus-maze and the shock-probe burying tests (Treit & Pesold, 1990). This anterior/posterior dissociation may seem somewhat curious in the context of the 'classical' neuroanatomical subdivisions of the septum. However, if the medial and lateral septal nuclei differentially modulate 'anxiety'-related behaviors, an anterior/posterior dissociation might be expected since the anterior septum is primarily composed of lateral septal nuclei, whereas the posterior septum contains both medial and lateral septal nuclei. Therefore, the anterior/posterior dissociation found in our laboratory may in fact reflect the differential role of the medial and lateral septal nuclei in the control of anxiety (Pesold, 1991). If this medial/lateral dissociation in the septum is true, then intracerebral infusions of midazolam into the medial septal nucleus might produce a different pattern of results than that of infusions into the lateral septum.

The purpose of this second experiment therefore was to examine the neuroanatomical specificity of the behavioral effects of intracerebral infusions of a benzodiazepine agonist into the septum, by comparing the effects of discrete infusions of midazolam into the medial and lateral septal nuclei, in both the plus-maze and the shock-probe burying tests. If these two septal nuclei differentially mediate the anxiolytic effects of benzodiazepines, then infusion of

midazolam into these two structures would be expected to produce different patterns of behavioral effects. Furthermore, if these effects are mediated via the benzodiazepine receptor, a pre-treatment with the benzodiazepine receptor antagonist Ro 15-1788, should block these effects.

METHOD

As in Experiment 1, the methods in Experiment 2 were the same as those described in the General Methods section with the exception of surgical coordinates and drug regimen.

Subjects

The subjects were 126 male, albino Sprague-Dawley rats, weighing 250-340 g at the time of surgery. Housing and feeding conditions were the same as those described in the General Methods section.

Surgery

Using flat skull coordinates, 60 rats were implanted with a guide cannula position 1.0 mm above the middle of the medial septal nucleus (0.50 mm anterior and 0.40 mm lateral to bregma, 4.90 mm ventral to dura, with the cannula angled 4 degrees medially to avoid the sagittal sinus), and 66 rats were bilaterally implanted with guide cannulae positioned 1.0 mm above the middle of the lateral septal nuclei (0.70 mm posterior and 2.60 mm lateral to bregma, 4.20 mm ventral to dura, with the cannula angled 22 degrees medially).

Procedures

Drug Pre-Treatment. Half of the medial septal-implanted animals were given a 0.5 μl infusion of flumazenil, and half were given a 0.5 μl infusion of vehicle. Half of the lateral septal-implanted animals were given 0.5 μl/side bilateral infusions of flumazenil, and the other half were given 0.5 μl/side bilateral infusions of vehicle. Infusion rate and diffusion time were the same as those described in the General Method section.

Drug Treatment. Ten minutes after drug pre-treatment, half of the flumazenil pre-treated animals received an infusion of midazolam (0.5 μl in the medial septum or 0.5 μl/side in the lateral septum), and the other half received an infusion of saline (0.5 μl in the medial septum or 0.5 μl/side in the lateral septum). Similarly, half of the vehicle pre-treated animals were infused with midazolam (0.5 μl in the medial septum or 0.5 μl/side in the lateral septum), while the other half were infused with saline (0.5 μl in the medial septum or 0.5 μl/side in the lateral septum).

RESULTS AND DISCUSSION

Histologies

The black dots in Figure 7 represent the location of the cannulae tips for the four groups of medial septum-implanted rats. The behavioral data from animals with their cannula located outside of the medial septum (n=11) were discarded leaving 12 rats in the VEH-SAL group, 12 rats in the VEH-MDZ group, 13 rats (12 in shock-probe) in the Ro-SAL group, and 12 rats in the Ro-MDZ group.

The location of the cannulae tips for the four groups of lateral septum-implanted rats are illustrated in Figure 8. Seven of the lateral septal-implanted rats had to be terminated due to illness before behavioral testing. The behavioral data from another 21 animals with at least one cannula located outside of the lateral septal nucleus were discarded leaving 12 rats in the VEH-SAL group, 8 rats in the VEH-MDZ group, 8 rats in the Ro-SAL group, and 10 rats in the Ro-MDZ group.

Plus-Maze

Figure 9 represents the mean percentage of time that the medial and lateral septal-infused rats spent in the open arms, and the mean percentage of entries that these rats made into the open arms of the plus-maze are shown in Figure 10. Clearly, the increase in open-arm activity observed in the

plus-maze in Experiment 1 was due to the effect of midazolam in the lateral septum.

Significant between groups analysis of variance [$F(7,79)=4.25$, $p<0.0007$] and Newman-Keuls pair-wise comparisons (alpha=0.05) confirmed that only animals that received midazolam in the lateral septum (VEH-MDZ) spent a significantly greater percentage of their time in the open arms compared to their respective controls (VEH-SAL). The increase in the percentage of time spent in the open arms by lateral-septal VEH-MDZ rats was blocked by a pre-infusion of Ro 15-1788, as the percentage of open-arm time of this group was significantly greater than all other groups (with the exception of the lateral-septal Ro-SAL group which showed a slight non-significant intrinsic effect), the latter seven groups did not differ significantly from each other. Similarly, only rats that received midazolam in the lateral septum (VEH-MDZ) had a significantly higher percentage of entries into the open arms than their respective controls [ANOVA, $F(7,79)=3.07$, $p<0.007$]. Newman-Keuls pair-wise comparisons (alpha=0.05) revealed that while the rats that had received either the antagonist alone (Ro-SAL), or the combination of both the agonist and the antagonist (Ro-MDZ) in the lateral septum did not display a significantly greater percentage of open-arm entries than their respective controls (VEH-SAL), their percentage of open-arm entries was not significantly lower than the rats that had only received

midazolam (VEH-MDZ). This finding suggests that the midazolam-induced increase in the percentage of open-arm entries observed in the lateral septal-infused rats, was not completely reversed by a pre-infusion of the antagonist Ro 15-1788. And while the percentage of open-arm entries of the lateral septum VEH-MDZ group was significantly different from all other groups (except for the lateral septum Ro-SAL and Ro-MDZ), these other seven groups were not significantly different from each other. These drug effects on open-arm activity in the lateral septum could not be attributed to any changes in general activity since there were no significant differences in total number of arm entries between any of the groups of rats [$F(7,79)=1.69$, $p>0.1$; see Table 4].

These results suggest that the lateral septal nuclei, not the medial septal nucleus, are mediating the anxiolytic effects of the benzodiazepine agonist midazolam in the plus-maze. Furthermore, despite the lack of complete antagonism of the percentage of open-arm entries by a pre-infusion of the benzodiazepine receptor blocker Ro 15-1788, it appears that these anxiolytic effects are being mediated, at least in part, via the benzodiazepine receptors in the lateral septum.

Shock-Probe Burying

Figure 11 shows the mean duration of time that the medial and the lateral septal-infused rats spent burying the shock-probe. Similar to the pilot data reported by Treit (1991),

midazolam did not produce any anxiolytic effect on burying when infused into the medial septum, however, infusions of midazolam into the lateral septum completely abolished burying behavior. Kruskal-Wallis one-way analysis of variance (H_{corr}=23.38; $p<0.001$) and Mann-Whitney U pair-wise comparisons revealed that only the group that received midazolam in the lateral septum (VEH-MDZ) had significantly lower burying levels than their respective controls (Mann-Whitney U=80.0, $p<0.005$). However, while the burying level of the lateral septum-implanted rats that received both drugs (Ro-MDZ) were not significantly different from that of their respective controls (VEH-SAL), they also did not differ significantly from the burying level of rats that only received midazolam (VEH-MDZ). These latter findings signify that the abolishment of burying behavior following midazolam infusions into the lateral septum was not completely reversed by a pre-infusion of the benzodiazepine antagonist. And while midazolam alone in the medial septum had no significant effect on burying behavior, pair-wise comparisons (Mann-Whitney U=117.5, $p<0.004$) revealed a significant suppression of burying in the medial septal rats that received the combination of the agonist and antagonist (Ro-MDZ). No other differences were significant (alpha=0.05). Overall, despite the lowered burying behavior in the animals that received the drug combination (Ro-MDZ) in the medial septum, these results suggest that the lateral septum, but not the medial septum, mediates the

anxiolytic effects of midazolam on burying behavior. Furthermore, the near significant reversal of these effects by the pre-administration of the antagonist suggests that these anxiolytic effects may be mediated, at least in part, by the benzodiazepine receptor.

The mean number of shocks that the medial and lateral septal-infused rats received from the probe are shown in Figure 12. In agreement with the results of Experiment 1, midazolam infusions into the septum had no effect on contact-induced probe-shocks. The behavioral effects of midazolam infusions into the lateral septum appear to be specific to burying behavior since there were no significant differences between any of the groups in terms of number of shocks obtained [$F(7,78)=0.89$, $p>0.5$], reactivity to those shocks [$F(7,78)=0.26$, $p>0.5$; see Table 5], or general activity levels [$F(7,78)=1.78$, $p>0.10$; see Table 5].

These results indicate that the lateral septal nucleus, but not the medial septal nucleus, mediates the anxiolytic effects of benzodiazepines on burying behavior, while neither of these septal nuclei mediate the anxiolytic effects of benzodiazepines on shock-probe avoidance.

Ataxia

As in experiment 1, central infusions of midazolam, Ro 15-1788, or their combination failed to produce any measurable ataxia in any of the animals.

Figure 7

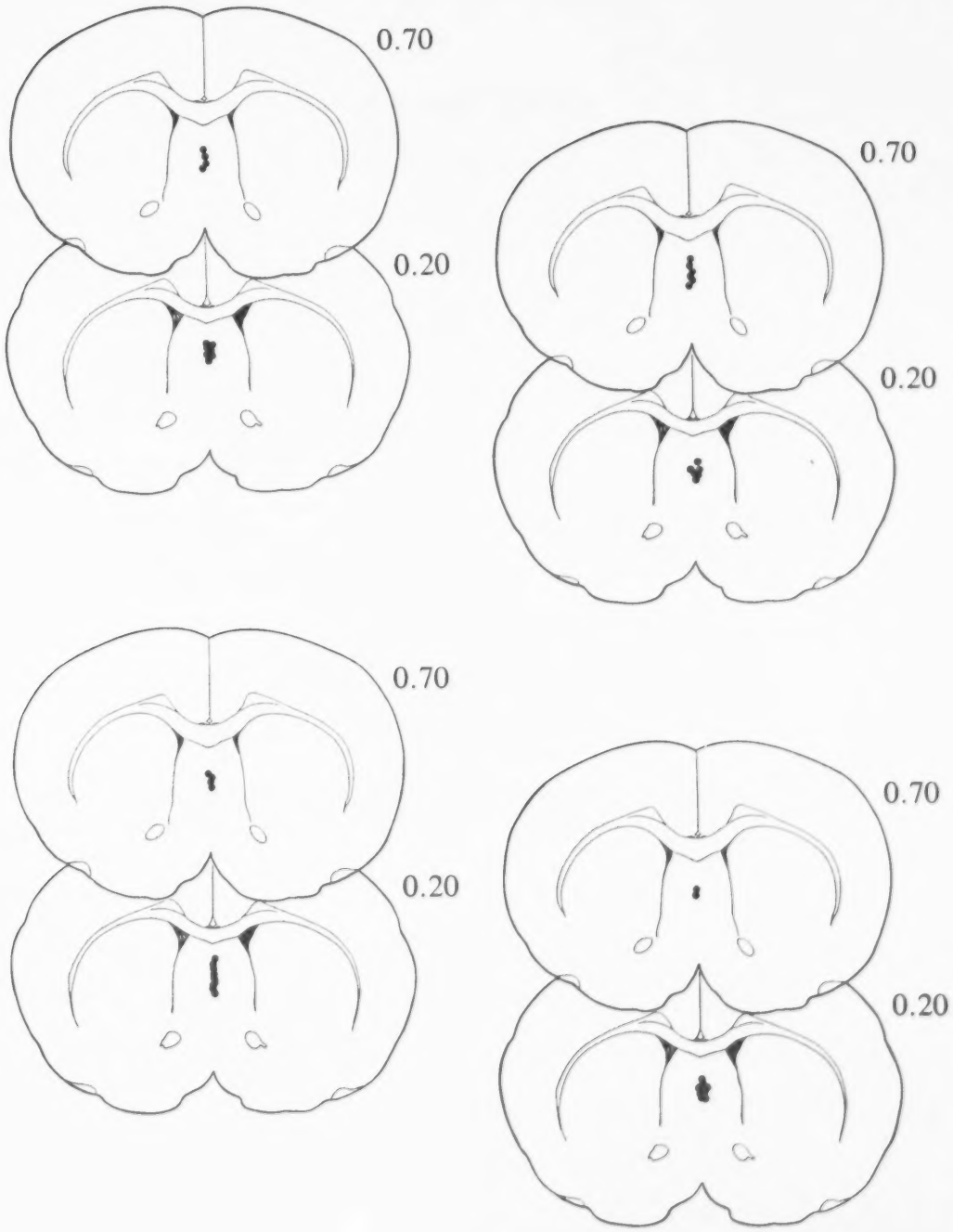


Figure 8

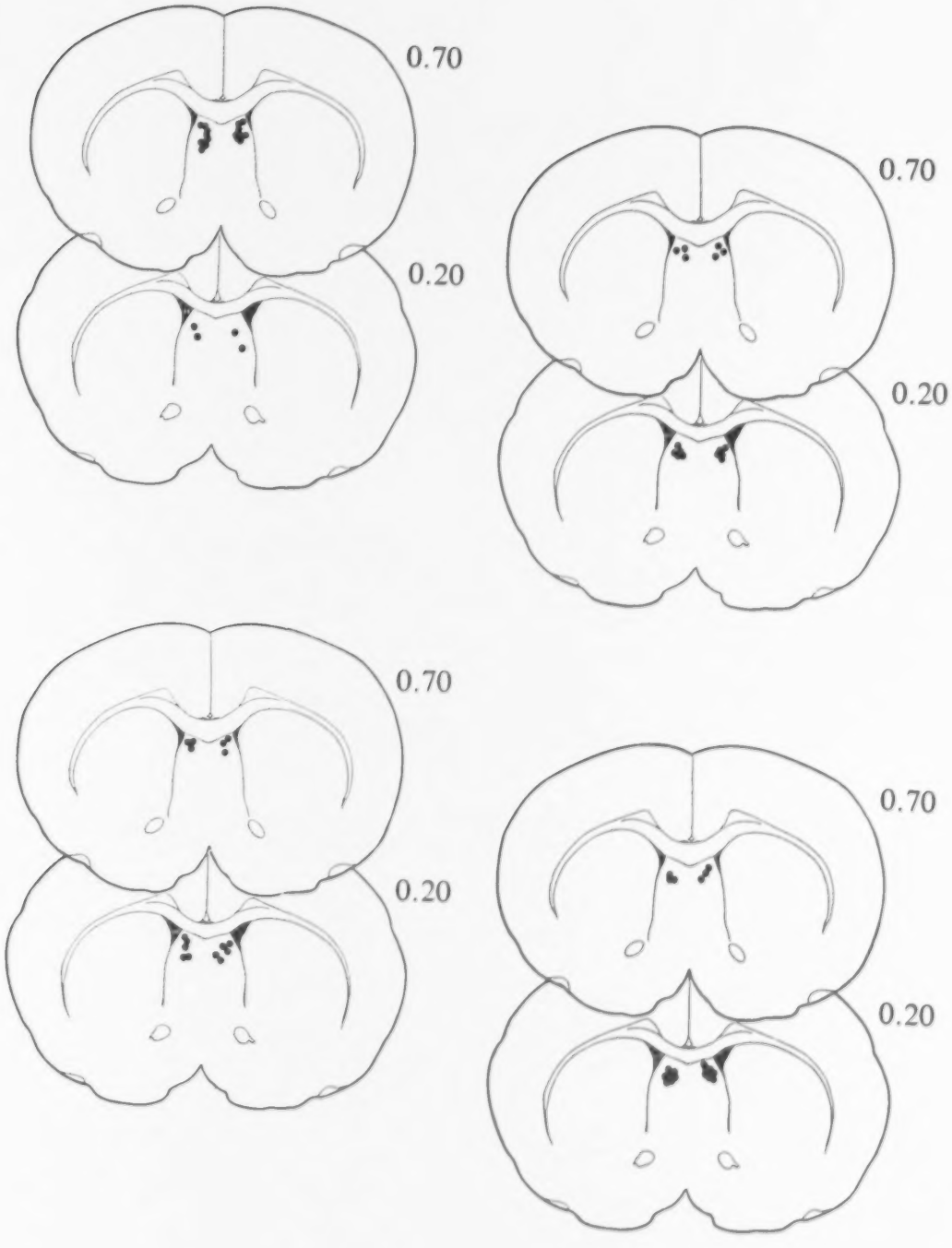
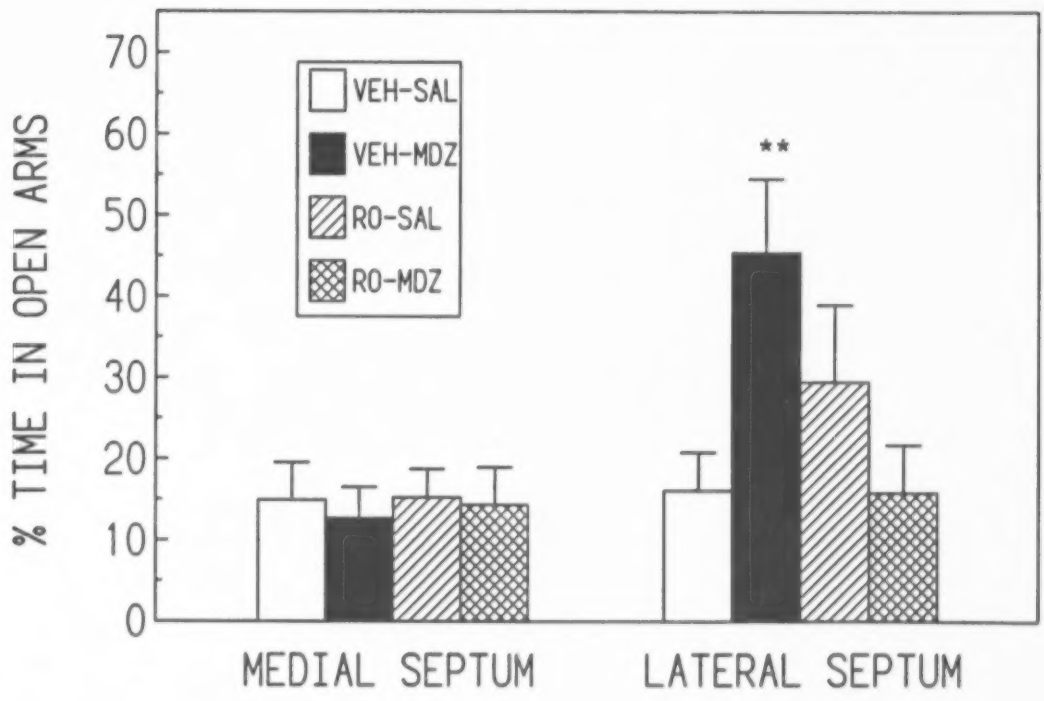
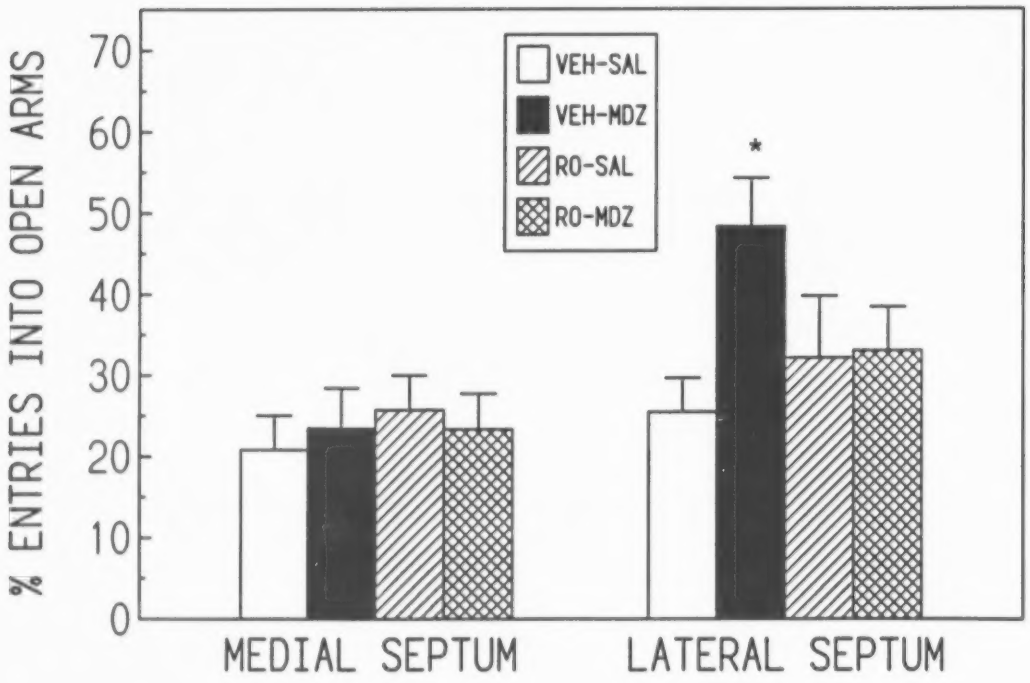


Figure 9



* p<0.05 compared to controls
** p<0.01 compared to controls

Figure 10



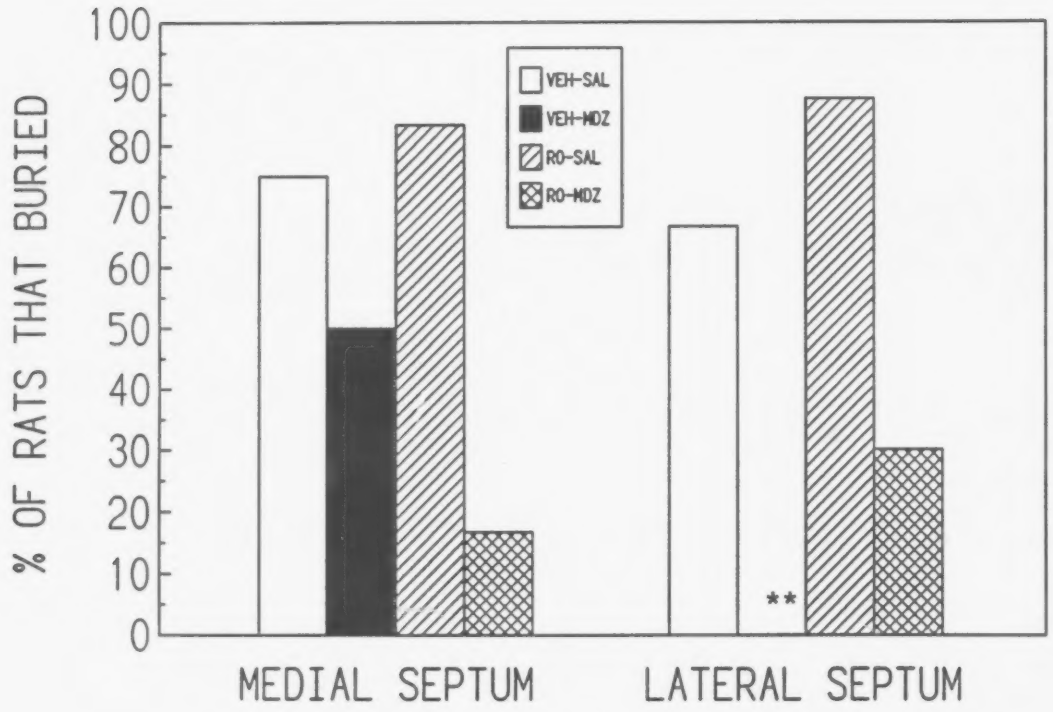
* p<0.05 compared to controls
** p<0.01 compared to controls

Table 4

		Total Number of Arm Entries
MEDIAL SEPTUM	VEH-SAL (n=12)	10.92 (0.85)
	VEH-MDZ (n=12)	10.00 (1.47)
	Ro-SAL (n=13)	11.31 (1.18)
	Ro-MDZ (n=12)	8.83 (1.40)
LATERAL SEPTUM	VEH-SAL (n=12)	9.67 (0.83)
	VEH-MDZ (n=8)	11.50 (1.81)
	Ro-SAL (n=8)	14.00 (1.24)
	Ro-MDZ (n=10)	9.00 (1.40)

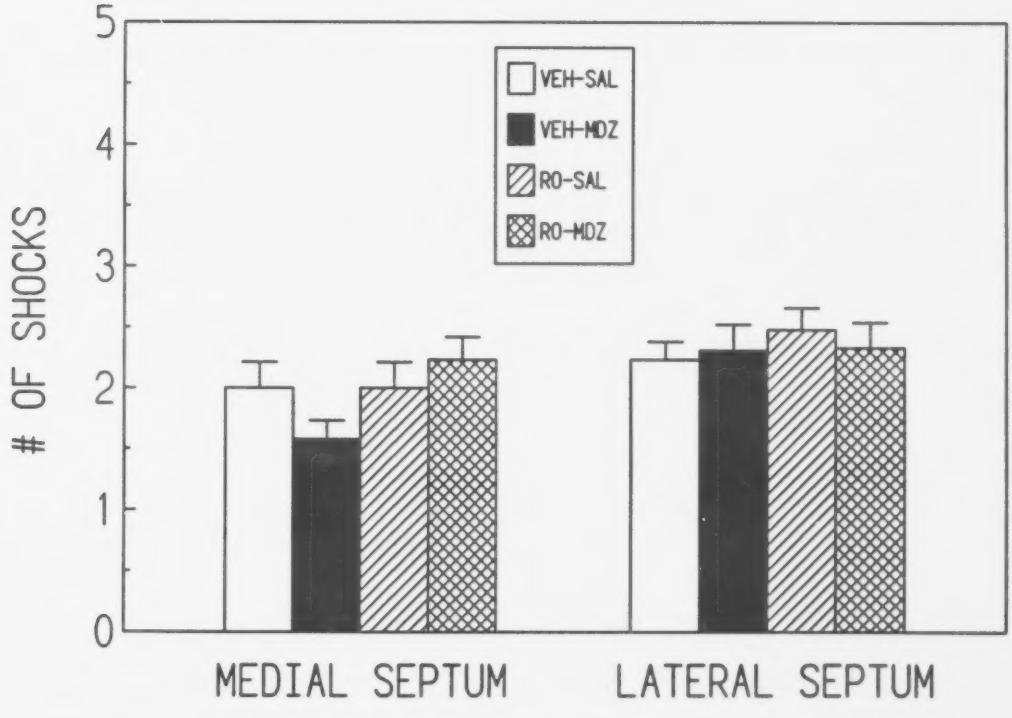
* $p<0.05$ compared to controls
** $p<0.01$ compared to controls

Figure 11



* p<0.05 compared to controls
** p<0.01 conpared to controls

Figure 12



* p<0.05 compared to controls
** p<0.01 compared to controls

Table 5

		Shock Reactivity	Immobility (sec)
MEDIAL SEPTUM	VEH-SAL (n=12)	2.27 (0.14)	139.17 (46.40)
	VEH-MDZ (n=12)	2.25 (0.20)	269.17 (61.24)
	Ro-SAL (n=12)	2.18 (0.11)	70.50 (17.87)
	Ro-MDZ (n=12)	2.23 (0.19)	248.25 (40.30)
LATERAL SEPTUM	VEH-SAL (n=12)	2.23 (0.15)	189.92 (54.11)
	VEH-MDZ (n=8)	2.31 (0.21)	130.63 (58.00)
	Ro-SAL (n=8)	2.48 (0.18)	158.88 (43.10)
	Ro-MDZ (n=10)	2.33 (0.21)	187.70 (45.43)

* $p<0.05$ compared to controls
** $p<0.01$ compared to controls

Experiment 3

Results from the first experiment suggested that the amygdala is not involved in the mediation of the anxiolytic effects of benzodiazepines on open-arm activity in the elevated plus-maze, or on burying behavior in the shock-probe burying test, since infusions of midazolam directly into the amygdala had no significant effect on either of these measures. However, the amygdala does appear to be mediating the anxiolytic effects of benzodiazepines on shock-probe avoidance since rats that received intra-amygdaloid infusions of midazolam also received significantly more shocks from the shock-probe than their respective controls.

The amygdala, however, is a complex of nuclei, each with different efferent and afferent projections. Many studies that have examined the behavioral effects of manipulating the subnuclei separately, have often noted quite different or even opposite effects, providing evidence that these subnuclei may differentially modulate 'anxiety'-related behaviors. For instance, lesioned-induced attenuation of gastric ulcers appeared to be specific to the central nucleus since lesions of the ventromedial and anterolateral nucleus had no effect, and lesions of the posterolateral regions actually potentiated the gastric ulcers (Henke, 1980). In addition to the neuroanatomical dissociations of anti-conflict effects observed following infusions of benzodiazepines into the

separate amygdaloid nuclei (Petersen et al., 1985; Shibata et al., 1982), anti-conflict effects have been observed following lesions of the central nucleus (Shibata et al., 1986), as well as lesions of the anterior basolateral nucleus, but not from lesions of the posterior regions of the basolateral nucleus (Shibata et al., 1989). Research that has examined the separate subnuclei have most consistently implicated the central or the basolateral nuclei in the modulation of 'anxiety'-related behaviors. Recent studies in our laboratory examined the effects of complete, as well as more circumscribed lesions of the amygdala in both the elevated plus-maze and the shock-probe burying tests (Treit et al., 1993a). While no amygdaloid lesions significantly affected behavior on the plus-maze, these studies indicated that lesions that included the central nucleus, tended to have a greater, albeit non-significant effect on burying behavior, as well as a significantly greater effect on number of shocks received, than lesions that were primarily restricted to the basolateral and lateral nuclei. In a recently published study, Green and Vale (1992) examined the behavioral effects of microinfusing midazolam into the central and basolateral nucleus of the amygdala in the elevated plus-maze test. While they did not find any effects of midazolam infusions into the central nucleus, they reported anxiolytic effects from basolateral infusions.

The purpose of this third experiment was to further

examine the neuroanatomical specificity of the anxiolytic effects of benzodiazepines in the amygdala, by comparing the effects of discrete infusions of midazolam into the central and the basolateral amygdaloid nuclei in both the plus-maze and shock-probe burying tests of anxiety. If these two amygdaloid nuclei differentially mediate the anxiolytic effects of benzodiazepines, then infusions of midazolam into these two nuclei should produce different patterns of behavioral effects. Furthermore, if these effects are mediated via the benzodiazepine receptor, they should be blocked by a pre-treatment with the benzodiazepine antagonist Ro 15-1788.

METHOD

As in the previous two experiments, methods in Experiment 3 were the same as those described in the General Methods section with the exception of surgical coordinates and drug regimen.

Subjects

The subjects were 123 male, albino Sprague-Dawley rats, weighing 255-335 g at the time of surgery. Housing and feeding conditions were the same as those described in the General Methods section.

Surgery

Using flat skull coordinates, 62 rats were bilaterally implanted with guide cannulae positioned 1.0 mm above the middle of the central amygdaloid nuclei (2.30 mm posterior and 4.40 mm lateral to bregma, 6.80 mm ventral to dura), and 61 rats were bilaterally implanted with guide cannulae positioned 1.0 mm above the middle of the basolateral nuclei (2.70 mm posterior and 5.10 mm lateral to bregma, 7.30 mm ventral to dura).

Procedures

Drug Pre-Treatment. Half of the central nucleus-implanted animals were given 0.5 μl/side bilateral infusions of flumazenil, and half were given 0.5 μl/side bilateral infusions of vehicle. Half of the basolateral nucleus-implanted animals were given 0.5 μl/side bilateral infusions of flumazenil, and the other half were given 0.5 μl/side bilateral infusions of vehicle. Infusion and diffusion rates were the same as those described in the General Methods section.

Drug Treatment. Ten minutes after drug pre-treatment, half of the flumazenil pre-treated animals received bilateral infusions of midazolam (0.5 μl/side), and the other half received bilateral infusions of saline (0.5 μl/side). Similarly, half of the vehicle pre-treated animals were infused with 0.5 μl/side midazolam, while the other half were infused with 0.5 μl/side saline.

RESULTS AND DISCUSSION

Histologies

In Figure 13, the black dots illustrate the location of the cannulae tips of the four groups of rats with central

amygdala placements. The behavioral data from animals with at least one cannula located outside of the central nucleus (n=12) were discarded leaving 13 rats in the VEH-SAL group, 13 rats in the VEH-MDZ group, 13 rats in the Ro-SAL group (12 for shock-probe), and 11 rats in the Ro-MDZ group (10 for shock-probe).

The black dots in Figure 14 represent the location of the cannulae tips for the four groups of rats with basolateral amygdala placements. Rats with at least one cannula located outside of the basolateral nucleus, or rats with extensive cannulae tracks that resulted in central amygdaloid nucleus lesions were discarded (n=11). One rat which was excluded from a central nucleus group for having misplaced cannulae was subsequently found to have near-perfect basolateral nucleus placements and was therefore included as a basolaterally-implanted rat, leaving 12 rats in the VEH-SAL group, 12 rats in the VEH-MDZ group, 13 rats in the Ro-SAL group, and 14 rats in the Ro-MDZ group.

Plus-Maze

The mean percentage of time that the central- and the basolateral-infused rats spent in the open arms of the plus-maze is illustrated in Figure 15, and the mean percentage of entries is illustrated in Figure 16. In agreement with the results of Green and Vale (1992), midazolam infusions into the basolateral nucleus produced a substantial increase in open-

arm activity in the plus-maze, whereas infusions into the central nucleus had no effect. Furthermore, these midazolam-induced increases in open-arm activity were completely antagonized by a pre-infusion of Ro 15-1788.

Significant between groups analysis of variance [$F(7,93)=4.21$, $p<0.0007$], and Newman-Keuls pair-wise comparisons (alpha=0.05) confirmed that only rats that had received infusions of midazolam into the basolateral nucleus (VEH-MDZ) spent a greater percentage of their time in the open arms of the plus-maze than their respective controls (VEH-SAL). Moreover, the percentage of open-arm time of the basolateral VEH-MDZ group was significantly greater than all other groups, the latter groups not differing significantly from each other. Similarly, analysis of variance [$F(7,93)=3.61$, $p<0.002$] and pair-wise comparisons (alpha=0.05) confirmed that only animals that had received basolateral infusions of midazolam showed a significant increase in percentage of entries into the open arms of the plus-maze compared to their respective controls. Like their percentage of open-arm time, the percentage of open-arm entries of the basolateral VEH-MDZ group was significantly greater than all other groups, and these groups were not significantly different from each other. While these results appear to be incongruent with those of Experiment 1 which showed that midazolam infused into the amygdaloid nucleus did not significantly increase open-arm activity in the plus-maze,

critical histological analysis revealed that the discrepancies between the two experiments may be due to the fact that the majority of the amygdala-implanted cannulae in Experiment 1 were located in the central nucleus. This latter finding strengthens the conclusion that the central nucleus is not involved in the mediation of the anxiolytic effects of benzodiazepines on open-arm avoidance in the plus-maze.

Analysis of variance also revealed a significant difference among the groups in their total number of arm entries [$F(7,93)=2.13$, $p<0.05$; see Table 6]. It is unlikely however, that these differences in general activity can account for the anxiolytic effects observed, since further analysis revealed that these differences were the result of a selective increase in open-arm entries in the basolateral VEH-MDZ group [$F(7,93)=3.78$, $p<0.001$; Newman-Keuls, alpha=0.05; see Table 6). None of the groups differed in their number of closed-arm entries [$F(7,93)=1.09$, $p>0.37$; see Table 6].

These results suggest that the basolateral, but not the central nucleus of the amygdala, mediates the anti-anxiety effects of benzodiazepine anxiolytics in the plus-maze. Furthermore, the complete blockade of this effect by the pre-infusion of a benzodiazepine antagonist suggests that these anxiolytic effects are mediated via the benzodiazepine receptor.

Shock-Probe Burying

The mean duration of time that the central- and basolateral-infused rats spent burying the shock-probe is illustrated in Figure 17. As depicted, midazolam, Ro 15-1788, or their combination had no significant effect on burying behavior when infused into either amygdaloid nuclei (Kruskal-Wallis H_{corr}=5.354; p>0.50; Mann-Whitney U, alpha=0.05). These findings are in agreement with those of Experiment 1, showing that burying behavior was not affected by intra-amygdaloid infusions of either of these drugs.

Midazolam did, however, have a significant effect on shock-probe avoidance when infused into the central, but not the basolateral nucleus of the amygdala (see Figure 18). Between groups ANOVA [F(7,91)=8.47, p<0.00001] and pair-wise comparisons (alpha=0.05) confirmed that only the rats that had received midazolam in the central nucleus (VEH-MDZ), received significantly more shocks than their respective controls (VEH-SAL). Subsequent pair-wise comparisons (Newman-Keuls, alpha=0.05) showed that this midazolam-induced increase in probe-contacts following central nucleus infusions was completely antagonized by the pre-infusion of the antagonist flumazenil, since this group made a significantly greater number of probe-contacts than all other groups, the latter groups not differing significantly from each other. This increased probe-contacts in the central VEH-MDZ rats could not be attributable to any changes in their sensitivity to shock,

since there were no significant differences in shock reactivity between any of the groups [$F(7,91)=1.66$, $p>0.13$; see Table 7]. And while analysis of variance revealed an overall difference in general activity levels [$F(7,91)=2.38$, $p<0.03$; see Table 7], it is unlikely that it contributed to the anxiolytic effect observed in the central VEH-MDZ group, since the immobility scores of these rats were not significantly different from any other group (Newman-Keuls, alpha=0.05).

Taken together, these results indicate that the central, but not the basolateral nucleus of the amygdala mediates the anxiolytic effects of benzodiazepines on shock-probe avoidance, whereas neither of these two amygdaloid nuclei mediate the anxiolytic effects of benzodiazepines on burying behavior. Furthermore, the complete reversal of the midazolam-induced increase in probe contacts by flumazenil indicated that this anxiolytic effect was mediated at the benzodiazepine receptor site.

Ataxia

As in Experiment's 1 and 2, infusions of midazolam, flumazenil, or their combination into either the central or the basolateral amygdaloid nuclei failed to produce any measurable ataxia.

Figure 13

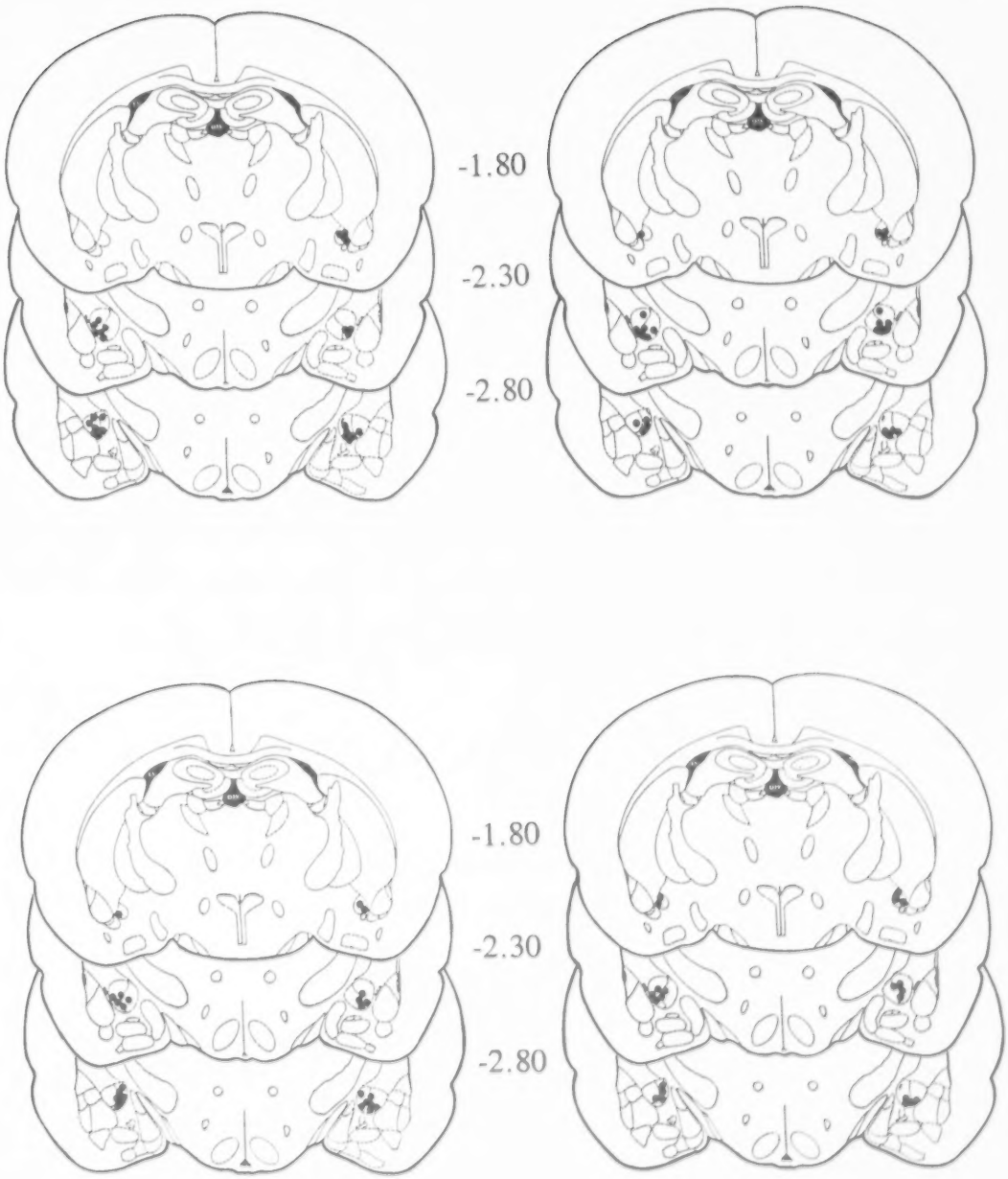
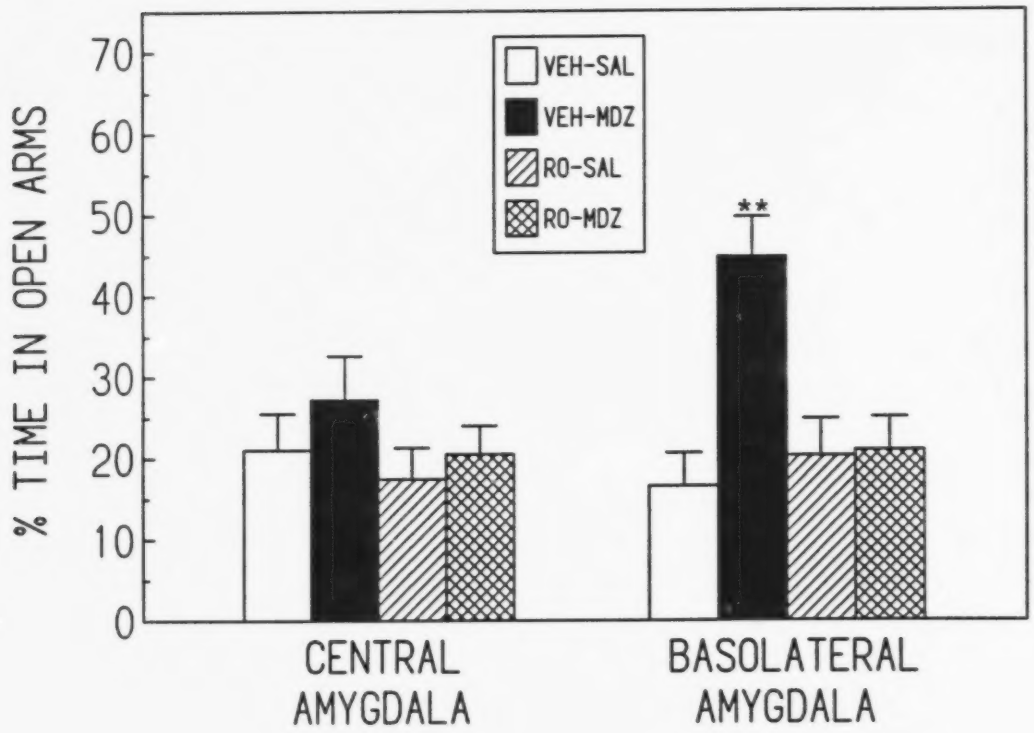


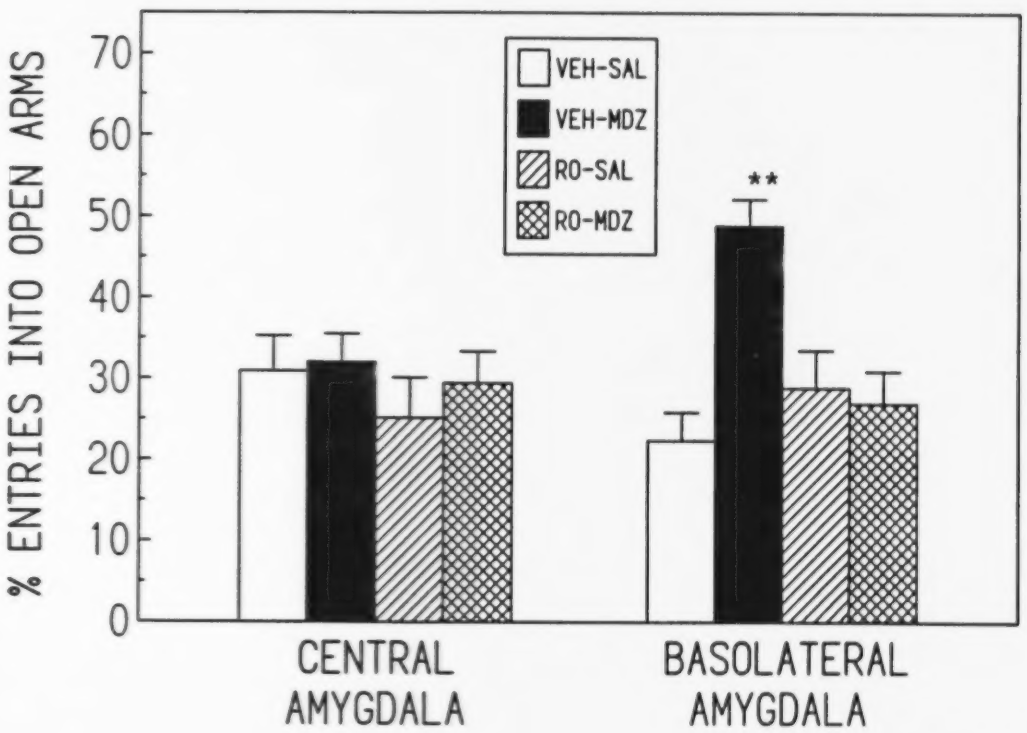
Figure 14

Figure 15



* p<0.05 compared to controls
** p<0.01 compared to controls

Figure 16



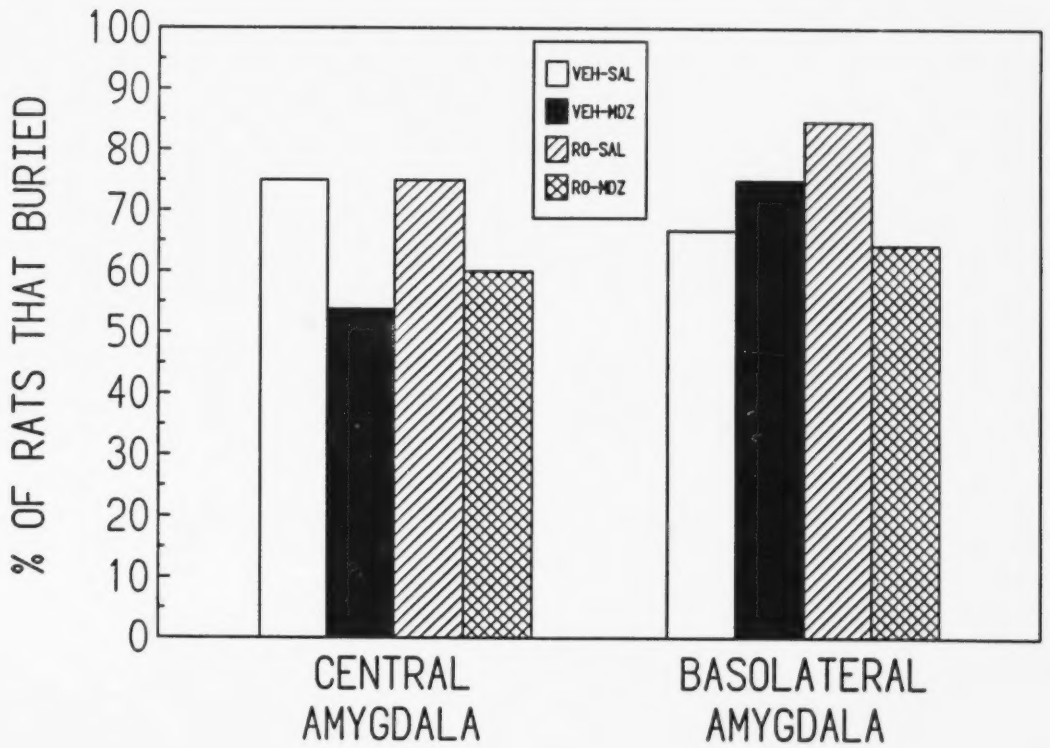
* p<0.05 compared to controls
** p<0.01 compared to controls

Table 6

		Number of Closed Arm Entries	Number of Open Arm Entries	Total Number of Arm Entries
CENTRAL AMYGDALA	VEH-SAL (n=13)	7.54 (0.84)	3.46 (0.68)	11.00 (1.04)
	VEH-MDZ (n=13)	9.38 (0.53)	4.77 (0.67)	14.15 (0.93)
	Ro-SAL (n=13)	8.00 (0.60)	2.85 (0.63)	10.85 (0.70)
	Ro-MDZ (n=11)	7.73 (0.84)	3.45 (0.69)	11.18 (1.35)
BASOLATERAL AMYGDALA	VEH-SAL (n=12)	7.83 (0.55)	2.50 (0.51)	10.33 (0.92)
	VEH-MDZ (n=12)	7.17 (0.80)	6.42 ** (0.66)	13.58 (1.20)
	Ro-SAL (n=13)	8.00 (0.63)	3.31 (0.60)	11.31 (0.51)
	Ro-MDZ (n=14)	8.57 (0.36)	3.57 (0.65)	12.14 (0.75)

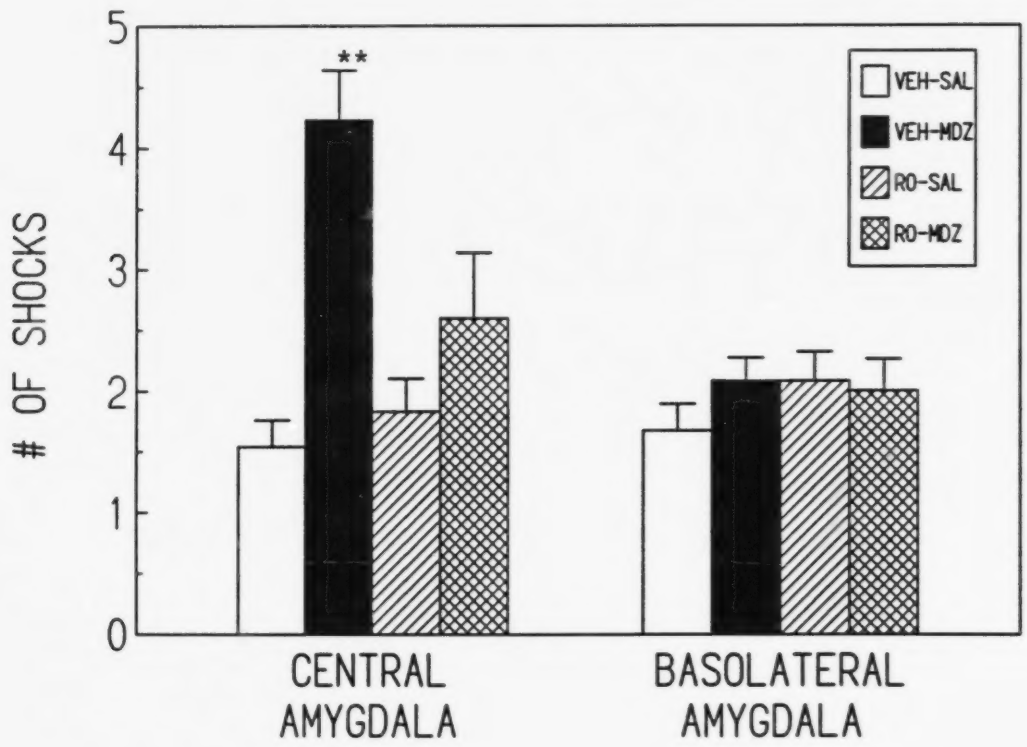
* $p<0.05$ compared to controls
** $p<0.01$ compared to controls

Figure 17



* p<0.05 compared to controls
** p<0.01 compared to controls

Figure 18



* p<0.05 compared to controls
** p<0.01 compared to controls

Table 7

		Shock Reactivity	Immobility (sec)
CENTRAL AMYGDALA	VEH-SAL (n=13)	2.31 (0.18)	98.54 (24.97)
	VEH-MDZ (n=13)	1.89 (0.11)	148.00 (23.02)
	Ro-SAL (n=12)	2.38 (0.19)	56.67 (13.13)
	Ro-MDZ (n=10)	2.02 (0.16)	147.50 (65.48)
BASOLATERAL AMYGDALA	VEH-SAL (n=12)	2.36 (0.15)	87.25 (25.60)
	VEH-MDZ (n=12)	2.35 (0.12)	240.33 (50.78)
	Ro-SAL (n=13)	2.42 (0.13)	100.23 (25.46)
	Ro-MDZ (n=14)	2.11 (0.15)	188.64 (56.51)

* $p<0.05$ compared to controls
** $p<0.01$ compared to controls

GENERAL DISCUSSION

The results of the present experiments support the hypothesis that the septum and the amygdala are differentially involved in the mediation of the anti-anxiety effects of benzodiazepine anxiolytics. In Experiment 1, microinfusions of midazolam directly into the septum and the amygdala had differing anti-anxiety effects in the elevated plus-maze and shock-probe burying tests. In the plus-maze, direct infusions of midazolam into the septum significantly increased open-arm activity but had no effect when infused into the amygdala. In the shock-probe burying paradigm, infusion of midazolam into the septum significantly decreased burying behavior without significantly affecting the number of shocks received from the probe. Conversely, infusion of midazolam into the amygdala produced a significant increase in the number of contact-induced probe-shocks, without having any significant effect on burying behavior. The anxiolytic effects of intra-septal midazolam on open-arm activity and burying behavior, as well as the anxiolytic effect of intra-amygdaloid midazolam on shock-probe avoidance, were mediated via the benzodiazepine receptor since these effects were blocked by the pre-administration of the benzodiazepine receptor antagonist flumazenil.

The results of Experiment 2 suggested that the anti-fear effects of intra-septal midazolam infusions in the first

experiment were a consequence of their actions in the lateral septum. Infusions of midazolam into the lateral septum significantly increased open-arm activity in the elevated plus-maze and abolished burying behavior in the shock-probe burying test, without having any significant effect on shock-probe avoidance. The anxiolytic effects of midazolam in the lateral septum appear to be due, at least in part, to the drug's action at the benzodiazepine receptor since the increase in percent time in the open-arms was completely reversed by the pre-administration of the antagonist Ro 15-1788, and the increase in percent entries into the open arms, as well as the abolishment of burying behavior in the shock-probe test, were partially reversed. In contrast, infusion of midazolam into the medial septum had no significant effect on any of these measures.

The results of Experiment 3 further characterized the neuroanatomical specificity of the anti-anxiety effects of intra-amygdaloid infusions of midazolam. In the plus-maze, infusions of midazolam into the basolateral, but not the central nuclei of the amygdala, had a profound anxiolytic effect on open-arm activity which was blocked by the pre-administration of flumazenil. Conversely, infusions of midazolam into the central, but not the basolateral nucleus, profoundly disrupted shock-probe avoidance, an effect which was also blocked by the antagonist, while infusions into neither structure had any significant effect on burying

behavior.

In accordance with the criteria outlined in the introduction, the results from these experiments provide further evidence that the septum and the amygdala are differentially mediating the anxiolytic effects of benzodiazepines. The anti-fear effects following central infusions of midazolam in these experiments, which were at least as large as those following systemic administration, were generally antagonized by the pre-administration of a benzodiazepine receptor antagonist. Furthermore, the pattern of anti-anxiety effects observed following benzodiazepine infusions in the first experiment was the same as those previously observed following lesions to these structures i.e., septal lesions increased open-arm activity in the elevated plus-maze and decreased burying behavior in the shock-probe burying paradigm, without affecting number of shocks obtained (Treit & Pesold, 1990; Pesold & Treit, 1992; Treit et al., 1993, 1993a), while amygdala lesions produced a profound effect on shock-probe avoidance, without producing any significant effect on open-arm activity or burying behavior (Kopchia et al., 1992; Roozendaal et al., 1991; Treit et al., 1993a, 1993b). Taken together, the results of these experiments provide converging evidence that the benzodiazepine anxiolytics are producing at least some of their anti-anxiety effects via the benzodiazepine receptors in the septum and the amygdala. Furthermore, the pattern of

results indicate that these two structures are differentially mediating these anti-anxiety effects.

While benzodiazepines, and physiological manipulations of both the septum and the amygdala, produce anxiolysis, they also produce a variety of other behavioral effects including changes in general activity, behavioral disinhibition, and memory deficits. In the following section, the results of the present experiments will be discussed in terms of these possible confounds, as well as potential problems that are inherent to the infusion technique employed. In the final sections, the role of the septum and the amygdala in anxiety, as well as the role of benzodiazepines in the modulation of anxiety will be discussed.

Alternative Interpretations

General Activity. While the benzodiazepines have potent anti-anxiety properties, they also have a variety of secondary properties including sedation, ataxia, and muscle relaxation, consequently producing changes in general activity when systemically administered. Physiological manipulations such as septal and amygdaloid lesions have also been shown to have various affects on general activity. Rats with septal lesions have been found to be hypoactive in some situations (e.g., Douglas & Raphelson, 1966; Trafton, 1967), while both rats with septal lesions (Nielson et al., 1965) and rats with

amygdaloid lesions (e.g., Schwartzbaum & Gay, 1966; Jellestad et al., 1986) have been found to be hyperactive in others. Therefore, since central infusions of midazolam in these two structures were not devoid of changes in general activity levels, the results of the present experiments must be examined as possible consequences of such a confound.

It is unlikely that the anxiolytic effects observed in the elevated plus-maze in these experiments are consequent to changes in general activity, since intra-septal infusions of midazolam in Experiment 1, as well as more discrete infusions of midazolam into the lateral septal nuclei in Experiment 2, were not accompanied by any significant changes in general activity (as measured by total number of arm entries). And while drug infusions in Experiment 3 appeared to produce significant differences in general activity between the groups, it is unlikely that these differences can account for the anxiolytic effects observed in the basolaterally- infused rats, since increased open-arm activity following infusions of midazolam into this nucleus appeared to be due to a selective increase in open-arm entries in this group.

In the shock-probe burying test, significant suppression of burying behavior was observed in rats that had received midazolam in the septum in Experiment 1. While this group of rats also had significantly lower general activity levels, it seems unlikely that their decrease in burying behavior was a result of this decrease in activity, since infusions of

midazolam into the lateral septum in Experiment 2 also abolished burying behavior but did not affect general activity levels.

The increase in probe-contacts observed following intracerebral infusions of midazolam into the amygdala in the first experiment, and following more discrete infusions into the central nucleus of the amygdala in the third experiment, also do not appear to be a result of changes in general activity, since activity levels of these two groups were no different from their respective controls.

In the present experiments, neither infusions of midazolam, flumazenil, nor their combination produced any ataxia when infused into either the septum, the amygdala, or any of their subnuclei. Therefore, while systemic administration of benzodiazepine agonists often produce a variety of 'side effects' such as ataxia, central administration of these same agents did not produce measurable ataxia in these experiments. It therefore seems reasonable to speculate, on the basis of the present results, that the anxiolytic effects of the benzodiazepines are a consequence of their ability to decrease neuronal activity in certain brain regions (e.g., limbic system structures), and that their sedative, ataxic, and muscle relaxant properties are a consequence of decreasing neuronal activity in other CNS regions (e.g., striatum, spinal cord). This notion is supported by the recent findings that only benzodiazepine

partial agonists which bind to the subclass of benzodiazepine receptors in the striatum tended to have sedative properties, while those that bind to the receptors found in the spinal cord tended to have ataxic properties (Guidotti et al., 1990).

Behavioral Disinhibition. The well-known propensity of septal-lesioned rats to over-respond in situations which require response inhibition has led some researchers to theorize that the septum is one structure in a system that controls behavioral inhibition (Gray, 1982). And similar to the effects of benzodiazepines, manipulations of the amygdala such as lesions and drug infusions, tend to produce behavioral effects that are also compatible with behavioral disinhibition e.g., impairments in passive avoidance, conditioned suppression, conditioned freezing, and response attenuation in conflict paradigms. In light of these data, the possibility that the results of the present experiments might be explained by a simple behavioral disinhibition must be considered.

The rats that were infused with midazolam into the septum in Experiment 1, and more discretely into the lateral septum in Experiment 2, displayed significantly higher open-arm activity in the plus-maze than their respective controls. While this increase in open-arm activity is consonant with behavioral 'disinhibition', it is unlikely that these rats were generally disinhibited given that they also showed significant reductions, or abolishment of burying behavior,

and no change in shock-probe avoidance in the shock-probe burying paradigm. Similarly, rats that received midazolam in the basolateral nucleus in Experiment 3 also showed an increase in open-arm activity, without any changes in burying behavior, or passive avoidance of the shock-probe. Finally, the rats that were impaired in their passive avoidance of the shock-probe (i.e., rats that received midazolam in the amygdala in the first experiment, and more specifically in the central nucleus of the amygdala in Experiment 3), were not impaired in their passive avoidance of the open-arms of the plus-maze, or in their active burying of the shock-probe.

In conclusion, it is difficult to explain the pattern of results obtained in the present investigations simply in terms of a non-specific 'behavioral disinhibition'.

Learning and Memory Deficits. Systemic administration of benzodiazepines have been reported to have amnesic effects in both animals and humans (Cole, 1986; Lister, 1985). Similarly, the amygdala (McGaugh et al., 1990; Sarter & Markowitsch, 1985), and the septum via its close interconnections with the hippocampus (Gray & McNaughton, 1983), have both been implicated in the modulation of learning and memory. Furthermore, intra-septal drug infusions have been found to directly impair memory (Brioni, Decker, Gamboa, Izquierdo, & McGaugh, 1990; Chrobak & Napier, 1992; Givens & Olton, 1990), whereas drugs infused into the amygdala have been found to

have their most profound effects on measures of conditioned avoidance, which rely on the integrity of the learning and memory system (e.g., Hodges et al., 1987). For these reasons, the results of the present experiments must be examined as possible consequences of learning and memory impairments.

The lack of a clear memory requirement in the elevated plus-maze makes it seem unlikely that a memory deficit could account for the increase in open-arm activity observed in the rats that received midazolam in the septum, the lateral septum, and the basolateral amygdala in these three experiments. Furthermore, the same rats that were anxiolytic in this test did not show any signs of memory impairments in the shock-probe burying test of anxiety i.e., they showed normal shock-probe avoidance.

The shock-probe burying test may have a greater memory requirement than the plus-maze test i.e., the rats had to remember that they had received a shock, as well as the location of the shock source. While rats that received infusions of midazolam into the septum in Experiment 1 and into the lateral septum in Experiment 2 showed significant reductions in burying behavior, they were not impaired in their ability to avoid the shock-probe. If these rats were simply unable to remember that they had been shocked (or the location of the shock-source), they would also not be expected to show the normal avoidance of the probe observed. Furthermore, rats that obtained significantly more shocks from

the probe (i.e., amygdala-infused rats in Experiment 1, and central amygdala-infused rats in Experiment 3), did not show any significant reductions in their burying behavior. If these increased probe-contacts were due to a disruption in learning or memory (i.e., the inability to establish the relationship between the electric-shock and the shock-probe), rats would not display well-directed burying behavior toward the shock-probe. Burying behavior in these rats was indistinguishable from that of controls. Although impairments in learning and memory cannot be completely ruled out, it seems unlikely that they can account for the anxiolytic effects observed in these three experiments.

Specificity of Drug-Infusion Effects. The present experiments showed that the septum and the amygdala differentially mediate the anxiolytic effects of benzodiazepine anxiolytics. While examining the behavioral effects of intra-cerebral drug infusion is conceptually a logical method for determining a drug's location of action, there are potential practical problems that must be considered. The results of the present series of investigations will be discussed in terms of some of the pharmacological and neuroanatomical problems inherent to this technique.

It is unlikely that the anxiolytic effects of the benzodiazepine agonist midazolam in these structures were

simply a result of non-specific stimulation or inhibition of cellular activity, since these behavioral effects were specifically blocked by a pre-administration of a benzodiazepine antagonist flumazenil. And while the flumazenil-antagonism of the anxiolytic effects of midazolam infused into the lateral septum on both percent entries into the open arms and burying behavior in Experiment 2 did not reach statistical significance, this may have only been a corollary of the small sample size of this particular group of rats.

The different pattern of anxiolytic activity of midazolam in the structures examined was probably not a factor of the differential density of benzodiazepine receptors in these structures. For instance, some studies have demonstrated, using quantitative light microscopic autoradiographic techniques, that the basolateral nucleus of the amygdala has a higher density of benzodiazepine receptors than the central nucleus (Niehoff & Kuhar, 1983). However, while midazolam was anxiolytic when infused into the basolateral nucleus in the plus-maze but not when infused into the central nucleus, the lack of anxiolysis in the centrally-infused rats in this test cannot be due to such quantitative differences in benzodiazepine receptors, since these latter rats were anxiolytic on shock-probe avoidance, while the basolateral-infused rats were not. The anxiolytic effects of midazolam observed following infusions into the lateral septum but not

the medial septum are also not likely due to differences in receptor density between these two nuclei, since autoradiographic studies actually revealed that there is a higher density of benzodiazepine receptors in the medial septum that in the lateral septum (Young & Kuhar, 1980).

Diffusion of the drugs into neighbouring areas is also not a likely contributor to the present findings. Drug infusion volumes were relatively small, ranging from 1.0 μl or 1.0 μl/side in the first experiment, to only 0.5 μl or 0.5 μl/side in Experiments 2 and 3. Autoradiographic studies have shown that a volume of 1.0 μl of chlordiazepoxide infused at a low rate only produces a radial spread of about 0.5 mm from the injection site (Myers, 1975). Since the drug volume in these experiments were small and infused at the slow rate of 1.0 μl/min, it is relatively safe to assume that there was no meaningful diffusion into other areas. It is also safe to assume that the drug effects in these rats were not a result of their diffusion into the lateral ventricles, since lateral septal-implanted animals whose cannulae were either placed in, or bordering the lateral ventricles, were discarded. Interestingly, the behavior of these 12 animals was no different than that of controls, indicating that the amount of drug that was sufficient to produce such profound anxiolytic effects in the lateral septum, was too minute to produce any effects when infused indiscretely into the brain via the lateral ventricles.

The results of the present experiments were also not consequent to non-specific neuronal damage. All of the drug-infused rats were compared to control rats which suffered similar cannula tract lesions. Furthermore, analyses of variance between all of the control groups (i.e., VEH-SAL) showed that they were not significantly different from one another on any measure (alpha=0.05). Moreover, animals which suffered cannula-tract damage to critical structures (e.g., damage to the central nucleus from cannulae aimed at the basolateral nucleus), were discarded from the analysis.

In summary, the anxiolytic effects observed in the present experiments following infusions of midazolam into discrete structures, were not likely artifacts of non-specific stimulation, differences in receptor density, diffusion, or neuronal damage.

The Role of the Septum and the Amygdala in Anxiety

While the results of the present set of experiments provide little insight into the etiology of anxiety disorders, they are highly significant in that they indicate that the central and the basolateral nuclei of the amygdala, as well as the lateral and medial nuclei of the septum, play quite distinctive roles in the mediation of the anxiolytic effects of benzodiazepines, and possibly in the modulation of anxiety.

The central nucleus appears to be involved in the

'passive avoidance' of 'painful' aversive or fearful stimuli, but not in the 'active avoidance' of such stimuli, nor in the 'passive avoidance' of 'non-painful' aversive or threatening stimuli. This interpretation of the role of the central nucleus is congruent with much of the research that has examined the role of this structure in anxiety. For instance, physiological manipulations of the central nucleus (such as lesions and drug infusions) have consistently reported "anti-anxiety" effects, when 'anxiety' was measured by the animals ability to passively avoid shock e.g., conditioned freezing (Jellestad & Bakke, 1985), conflict (e.g., Shibata et al., 1986), potentiated startle (e.g., Hitchcock & Davis, 1986). However, when the measure of 'anxiety' did not involve the passive avoidance of shock, central-amygdala manipulations do not appear to be anxiolytic e.g., social interaction (Jonason & Enloe, 1971), elevated plus-maze (Treit et al., 1993a, 1993b), simple acoustic startle (Melia et al., 1991), neophobia (Becker et al., 1980). Further support for this hypothesis comes from studies that have directly compared the effects of amygdaloid lesions on fear reactions to different stimuli. For example, Cahill and McGaugh (1990) found that amygdaloid-lesioned rats showed impaired aversive learning when the aversive stimulus was footshock, but not when it was an aversive quinine solution. Furthermore, the impaired ability of these rats to learn to avoid shock is not a consequence of a simple decrease in shock-sensitivity since

traditional tests of analgesia have consistently failed to show changes in pain threshold after amygdaloid lesions (Cahill & McGaugh, 1990; Grijalva, Levin, Morgan, Roland, & Martin, 1990; Helmstetter, 1992).

While the results of the present experiments indicate that the central nucleus appears to be involved in the 'passive avoidance' of 'painful' aversive stimuli such as shock, the basolateral nucleus appears to be involved in the 'passive avoidance' of 'non-painful' aversive or fearful stimuli. The basolateral nucleus has been less extensively studied than the central nucleus, presumably since it has also been less consistently found to be involved in the modulation of experimental anxiety. This is not surprising given that most tests that measure experimental anxiety in animals involve some variation of behavioral responses (usually passive avoidance) to shock. The differential role of these two structures in anxiety is also not surprising given their distinctive neuroanatomical connections. The lateral and basolateral nuclei of the amygdala appear to be the "sensory gateway" to the amygdala. The lateral nucleus sends projections to the basolateral nucleus, which together receive input from all sensory modalities (Ledoux, Cicchetti, Xagoraris, & Romanski, 1990). The basolateral nucleus, in turn, sends projections to the central nucleus (Krettek & Price, 1978), as well as to the neocortex (Amaral & Price , 1984). The central nucleus has extensive efferent and afferent

connections with the brainstem nuclei that are involved in the modulation of pain [i.e., the periaqueductal gray (Beart, Summers, Stephenson, Cook, & Christie, 1990; Nauta & Domesick, 1982)]. It is therefore possible that the central nucleus, via its connections with the PAG, modulates anxiety, or more specifically 'passive avoidance' to 'painful' aversive stimuli. In contrast, the aversive or fearful nature of 'non-painful' stimuli may be more subtle and more complex than 'painfully' aversive stimuli. For instance, the interpretation of the threatening nature of 'non-painful' stimuli such an open elevated platform, may not only require information from different sensory modalities, it may also require some 'cognitive interpretation'. The basolateral nucleus, which receives information from all sensory modalities, may therefore be mediating anxiety, or more specifically 'passive avoidance' of such 'non-painful' aversive or threatening stimuli via its connections with the neocortex.

In the present experiments, the septum, and more specifically the lateral septum, was also found to modulate 'passive avoidance' to 'non-painful' fearful stimuli. This interpretation of the role of the septum in anxiety is compatible with Gray's (1982) behavioral inhibition theory, which essentially views the septum as part of a neural system that inhibits behavior in response to novel or innate fear stimuli. Furthermore, it is also consistent with much of the research that has examined the effects of septal manipulations

in tests of 'anxiety' that do not involve shock (e.g., social interaction, elevated plus-maze, neophobia). The apparent similarities between the role of the basolateral nucleus of the amygdala and that of the lateral nucleus of the septum in the modulation of passive avoidance responses to non-painful stimuli, are strengthened by their neuroanatomical connections. The lateral septum has extensive direct interconnections with the basolateral amygdala (Shiosaka et al., 1983). In addition, the lateral septum also has extensive indirect connections with this nucleus via the neocortex and the hippocampus i.e., the areas of the neocortex that receive afferents from the basolateral nucleus, send projections to the hippocampus (Krettek & Price, 1978), a structure which is closely interconnected with the septum. The subicular area of the hippocampus, in turn, has extensive projections back to the basolateral nucleus (Ottersen, 1982). It is possible therefore that the septum and the basolateral nucleus of the amygdala may work in concert to mediate passive avoidance responses to non-painful threatening or fearful stimuli. While the present experiments cannot verify this postulate, the results do however indicate that the septum, or more specifically the lateral septum, is also involved in the mediation of the 'active avoidance' (e.g., burying) of painful aversive stimuli (e.g., electric shock). While it appears from the present results that the amygdala is not involved in the modulation of active responses to painful stimuli, the neural

system via which the septum mediates these effects is as yet unknown.

The lack of behavioral effect following benzodiazepine infusions into the medial septum is not extremely surprising in light of the apparent dissimilarities in the effects of lesioning the medial and lateral septal nuclei. However, given that the lateral septum tends to have inhibitory modulatory effects over the activity of medial septal neurons (Thomas, 1988), infusions of these drugs into the medial septum might be expected to produce behavioral effects that are opposite to those following lateral septal infusions (i.e., anxiogenesis). Although counter-intuitive, the results of Experiment 2 are not incompatible with such a conclusion. For instance, anxiogenic effects following medial septal-infusions of midazolam in these tests may have been masked by 'floor effects' on both open-arm activity in the plus-maze, and shock-probe avoidance in the shock-probe burying test. And while these rats did not exhibit an increase in burying behavior, they did spend considerably more time immobile, a behavior which may be both indicative of fear and incompatible with active responses such as burying. Therefore, while the results of the present experiments do not support an anxiogenic role for the medial septum, such a role cannot be ruled out.

In summary, the central nucleus, with its extensive interconnections with brainstem nuclei, appears to be involved

in the modulation of 'passive' avoidance responses to 'painful' stimuli. The basolateral amygdala, presumably via its inputs from sensory modalities and its connections to the neocortex, appears to be involved in the modulation of 'passive' avoidance responses to 'non-painful' fear stimuli. The lateral septum also appears to be involved in the modulation of 'passive' avoidance responses to 'non-painful' fear stimuli, presumably via its direct and indirect connections with the basolateral nucleus, as well as in the modulation of 'active' responses to 'painful' stimuli. And finally, while the results of the present experiments do not support a role for the medial septum in the mediation of benzodiazepines' anxiolytic effects, it cannot be ruled out.

The Role of Benzodiazepines in the Modulation of Anxiety

While there is no complete theory of the biological basis of anxiety, there is considerable evidence that anxiety may be primarily modulated by limbic system structures, including the septum and the amygdala. The activity of these heavily interconnected structures is presumed to be modulated by serotonergic and noradrenergic inputs from such brainstem structures as the raphe nuclei and the locus coeruleus, respectively (for reviews, see Davis, 1992b; Graeff, 1990; Gray, 1991; and Panksepp, 1990). Interestingly, while benzodiazepine receptors are present in many areas of the

central nervous system, they seem to be in particularly high abundance in the aforementioned structures. The presence of these receptors in the brain, however, suggests that there must be endogenous ligand(s) that naturally react with these benzodiazepine receptors. While the search for such endogenous benzodiazepine receptor ligands has as yet been unsuccessful (De Robertis, Pena, Paladini, & Medina, 1988), their existence is supported by evidence that there are differences in the binding characteristics of "benzodiazepine-like molecules" in the brain of rats following stressful or fearful conditions (e.g., Wolfman et al., 1991; Da Cunha et al., 1992). Interestingly, these changes in the binding characteristics of these ligands are primarily found in limbic system structures including the septum and the amygdala.

It seems reasonable to speculate, therefore, that there may be endogenous benzodiazepine-receptor ligands (agonist and/or inverse agonists) that are released in response to stressful or fearful situations. And depending on the nature of the fearful stimulus, as well as the appropriate fear response in the given situation, these ligands would then bind to a selective population of benzodiazepine receptors located in the appropriate structure(s) of the neural circuitry, and hence modulate the activity of these structures.

Concluding Comments

The results of the present experiments show that the central and basolateral nuclei of the amygdala, as well as the lateral and medial nuclei of the septum, play quite distinctive roles in the mediation of the anxiolytic effects of benzodiazepines, and possibly in the modulation of anxiety. These findings underline the importance of changing our current understanding of "anxiety" as a unitary concept. The heterogeneous nature of human anxiety and anxiety disorders, coupled with the failure of benzodiazepine anxiolytics to be therapeutically effective in all forms of anxiety disorders, suggests that there are many systems involved in the control of anxiety, not all of which are modulated by benzodiazepine ligands. A new direction of research, therefore, that will dissect and investigate particular behavioral reactions to specific types of fear-inducing stimuli, may be more fruitful in our understanding of the neural substrates of anxiety.

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APPENDIX

Table 1. The effects of intra-amygdaloid $GABA_A$/benzodiazepine receptor ligands on conflict behavior in the rat

Drug	Dose	Structure	Effect
	Nagy et al., 1979		
Diazepam (DZ)	25 μg/2 μl (i.c.)	amygdala	0
	50 μg/2 μl (i.c.)	amygdala	++
	100 μg/2 μl (i.c.)	amygdala	+++
	Shibata et al., 1982		
Diazepam	20 μg/1 μl (i.c.)	Central n.	++
Chlordiazepoxide	60 μg/1 μl (i.c.)	Central n.	++
Midazolam	30 μg/1 μl (i.c.)	Central n.	++
Diazepam	20 μg/1 μl (i.c.)	Medial n.	0
Chlordiazepoxide	60 μg/1 μl (i.c.)	Medial n.	0
Midazolam	30 μg/1 μl (i.c.)	Medial n.	0
Diazepam	20 μg/1 μl (i.c.)	Basolat n.	0
Chlordiazepoxide	60 μg/1 μl (i.c.)	Basolat n.	0
Midazolam	30 μg/1 μl (i.c.)	Basolat n.	0
	Scheel-Kruger et al., 1982		
Midazolam	1.0 μg/0.5 μl (i.c.)	Central n.	0
Midazolam	0.1 μg/0.5 μl (i.c.)	med Lat/BL	0
Midazolam	1.0 μg/0.5 μl (i.c.)	ant Lat/BL	0
Midazolam	1.0 μg/0.5 μl (i.c.)	post Lat/BL	0
Midazolam	1.0 μg/0.5 μl (i.c.)	med Lat/BL	+++
Midazolam	1.0 μg/0.5 μl (i.c.)	med Lat/BL	
BMI	0.25 μg/0.5 μl (i.c)	med Lat/BL	0
Muscimol	25 ng/0.5 μl (i.c.)	med Lat/BL	+++
Muscimol	25 ng/0.5 μl (i.c.)	med Lat/BL	
BMI	0.25 μg/0.5 μl (i.c)	med Lat/BL	0
Diazepam	1.0 μg/0.5 μl (i.c.)	med Lat/BL	+++

Table 1. (Continued)

Drug	Dose	Structure	Effect
	Petersen et al., 1985		
Midazolam	1.0 μg/0.5 μl (i.c.)	ant Lat/BL	+++
Midazolam + Ro 15-1788	1.0 μg/0.5 μl (i.c.) + 30 mg/kg (p.o.)	ant Lat/BL	+
Midazolam + Ro 15-1788	1.0 μg/0.5 μl (i.c.) + 60 mg/kg (p.o.)	ant Lat/BL	0
Midazolam + ZK 93426	1.0 μg/0.5 μl (i.c.) + 1 mg/kg (i.p.)	ant Lat/BL	++
Midazolam + ZK 93426	1.0 μg/0.5 μl (i.c.) + 3 mg/kg (i.p.)	ant Lat/BL	++
Midazolam + ZK 93426	1.0 μg/0.5 μl (i.c.) + 10 mg/kg (i.p.)	ant Lat/BL	0
Midazolam + FG 7142	1.0 μg/0.5 μl (i.c.) + 10 mg/kg (i.p.)	ant Lat/BL	0
Midazolam + CGS 8216	1.0 μg/0.5 μl (i.c.) + 1 mg/kg (i.p.)	ant Lat/BL	+
Midazolam + CGS 8216	1.0 μg/0.5 μl (i.c.) + 2 mg/kg (i.p.)	ant Lat/BL	0
	Thomas et al., 1985		
Chlordiazepoxide	10 μg/1 μl (i.c.)	Lateral n.	+++
	Kataoka et al., 1987		
GABA	30 μg/1 μl (i.c.)	Central n.	++
GABA	70 μg/1 μl (i.c.)	Central n.	+
Muscimol	0.01 μg/1 μl (i.c.)	Central n.	+
Muscimol	0.03 μg/1 μl (i.c.)	Central n.	+
	Takao et al., 1992		
d-AP159	30 μg/1 μl (i.c.)	Central	+
d-AP159 + Ro 15-1788	30 μg/1 μl (i.c.) + 10 mg/kg (i.p.)	Central	0

Table 1. (Continued)

Drug	Dose	Structure	Effect
	Hodges et al., 1987		
Midazolam	1.0 μg/0.5 μl (i.c.)	Lat/BL	+++
Chlordiazepoxide	10 μg/0.5 μl (i.c.)	Lat/BL	+++
Chlordiazepoxide	10 μg/0.5 μl (i.c.)	Lat/BL	
Ro 15-1788	10 μg/0.5 μl (i.c.)	Lat/BL	0
Chlordiazepoxide	10 μg/0.5 μl (i.c.)	Lat/BL	
Ro 15-1788	5 mg/kg (i.p.)		0
Chlordiazepoxide	10 mg/kg (i.p.)		
Ro 15-1788	10 μg/0.5 μl (i.c.)	Lat/BL	+
Chlordiazepoxide	10 μg/0.5 μl (i.c.)	Lat/BL	
CGS 8216	2 μg/0.5 μl (i.c.)	Lat/BL	0
Chlordiazepoxide	10 μg/0.5 μl (i.c.)	Lat/BL	
CGS 8216	5 mg/kg (i.p.)		0
Chlordiazepoxide	5 mg/kg (i.p.)		
CGS 8216	2 μg/0.5 μl (i.c.)	Lat/BL	0
Chlordiazepoxide	10 μg/0.5 μl (i.c.)	Lat/BL	
FG 7142	10 μg/0.5 μl (i.c.)	Lat/BL	0
Chlordiazepoxide	10 μg/0.5 μl (i.c.)	Lat/BL	
FG 7142	5 mg/kg (i.p.)		0
Chlordiazepoxide	5 mg/kg (i.p.)		
FG 7142	10 μg/0.5 μl (i.c.)	Lat/BL	0
GABA	500 ng/0.5 μl (i.c.)	Lat/BL	+++
GABA	500 ng/0.5 μl (i.c.)	Lat/BL	
Ro 15-1788	10 μg/0.5 μl (i.c.)	Lat/BL	+++
GABA	500 ng/0.5 μl (i.c.)	Lat/BL	
CGS 8216	2 μg/0.5 μl (i.c.)	Lat/BL	0
GABA	500 ng/0.5 μl (i.c.)	Lat/BL	
FG 7142	10 μg/0.5 μl (i.c.)	Lat/BL	0
Ro 15-1788	10 μg/0.5 μl (i.c.)	Lat/BL	-
CGS 8216	2 μg/0.5 μl (i.c.)	Lat/BL	0
FG 7142	10 μg/0.5 μl (i.c.)	Lat/BL	0
	Higgins et al., 1991		
Flurazepam	200 ng/1.0 μl (i.c.)	Central n.	++

Table 1. (Continued)

Drug	Dose	Structure	Effect
	Shibata et al., 1989		
Diazepam	5 μg/2 μl (i.c.)	ant Ce	0
Diazepam	10 μg/2 μl (i.c.)	ant Ce	0
Diazepam	20 μg/2 μl (i.c.)	ant Ce	++
Diazepam Ro 15-1788	20 μg/2 μl (i.c.) 20 mg/kg (i.p.)	ant Ce	0
Diazepam β-CCM	20 μg/2 μl (i.c.) 5 mg/kg (i.p.)	ant Ce	0
Zopiclone	2 μg/2 μl (i.c.)	ant Ce	0
Zopiclone	5 μg/2 μl (i.c.)	ant Ce	0
Zopiclone	10 μg/2 μl (i.c.)	ant Ce	+
Zopiclone Ro 15-1788	10 μg/2 μl (i.c.) 20 mg/kg (i.p.)	ant Ce	0
Zopiclone β-CCM	10 μg/2 μl (i.c.) 5 mg/kg (i.p.)	ant Ce	0
Phenobarbital	10 μg/2 μl (i.c.)	ant Ce	0
Phenobarbital	20 μg/2 μl (i.c.)	ant Ce	0
Phenobarbital	40 μg/2 μl (i.c.)	ant Ce	++
Phenobarbital Ro 15-1788	40 μg/2 μl (i.c.) 20 mg/kg (i.p.)	ant Ce	++
Phenobarbital β-CCM	40 μg/2 μl (i.c.) 10 mg/kg (i.p.)	ant Ce	0
Flurazepam	40 μg/2 μl (i.c.)	ant Ce	0
Flurazepam	80 μg/2 μl (i.c.)	ant Ce	0
Lormetazepam	2 μg/2 μl (i.c.)	ant Ce	0
Lormetazepam	5 μg/2 μl (i.c.)	ant Ce	0
Lormetazepam	10 μg/2 μl (i.c.)	ant Ce	++

0 = no effect
- = anxiogenic effect
+ = anxiolytic effect ($p<0.05$)
++ = anxiolytic effect ($p<0.01$)
+++ = anxiolytic effect ($p<0.001$)